INACTIVATION OF FOODBORNE PATHOGENS DURING CIDER FERMENTATION, IN A CIDER MODEL SYSTEM AND COMMERICAL CIDER

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ABSTRACT

Inactivation of Foodborne Pathogens During Cider Fermentation,

in a Cider Model System and Commercial Cider

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Hard cider is an alcoholic drink made from fermented crushed fruit, typically apples. The popularity of this fermented alcoholic beverage has been on the rise within the last decade. Historically, hard cider has been deemed safe due to the presence of ethanol and the low pH. Although there is lack of scientific evidence to prove that hard cider will and can be safe from foodborne pathogens. *Escherichia coli* O157:H7, *Salmonella* spp., and *Listeria monocytogenes* are three predominate foodborne bacterial pathogens of concern in the food and beverage industry. *Escherichia coli* O157:H7 in particular has been associated with fresh produce and more specifically apples, and apple products such as apple juice. The purpose of this study was to determine the bactericidal effects of pH, ethanol, and malic acid on *Escherichia coli* O157:H7, *Salmonella* spp., and *Listeria monocytogenes* to evaluate the safety parameters for safe hard cider production and storage.

The fate of foodborne pathogens in cider was determined during hard cider fermentation, in a cider model system, and in commercial cider. *Escherichia coli* O157:H7, *Salmonella* spp, and *Listeria monocytogenes* did not survive a 5-day fermentation period resulting in a > 7 log CFU/mL reduction of each pathogen with no significant change in pH. The final ABV of the cider at the end of the 5-day fermentation was 4.4%. In the cider model system, the lower the pH and higher the ABV the quicker

die off was observed, at pH 2.8, 3.0, 3.2, and 3.4 with 7, 8, and 9% ethanol concentration there was a 6.6 log reduction in *E. coli* O157:H7 population after 1 day. By the 7-day incubation period, no pathogens were detected at all pH and ABV combinations except for at pH 3.6 and 3.8 with 4% ethanol having ≤0.6 log CFU/mL of the population surviving. Similar *E. coli* O157:H7 inactivation patterns were observed in the model system and in the commercial ciders. The six commercial ciders observed had varying pH, ABV (%), and malic acid concentrations but successfully resulted in a > 6 log CFU/mL reduction in population of *E. coli* O157:H7 within 4 days of incubation. The ciders with the highest ABV's, 8.7 and 9.6% observed a > 6 log reduction by 1 day. It was observed that at some point in time pH plays a bigger role in the presence of less ethanol, but it is clear that ethanol and pH work synergistically to kill of pathogens present in cider fermentation, a cider model, and commercial cider.

Keywords: Cider, Apple Juice, Foodborne Pathogens, Ethanol

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1. INTRODUCTION

Hard cider is one of the oldest beverages known in history, found in documents dating to ancient times (National Apple Museum). Historically, cider was produced in England, France, Spain, and other European countries. In the 18th and 19th centuries, this alcoholic beverage was widely consumed in the United States after being brought from England. During Prohibition, cider consumption declined when cider orchards were burned down (Rupp, 2015). Consequently, most apple growers planted sweeter apples largely unfit for alcoholic cider. By the mid-1990s, hard cider sales rose because of growing interest in sweet alcoholic beverages (Keck, 2012). The highest rates of cider production and consumption remain in Europe, although consumption rates are rising in the United States. The term "cider" in the United States refers to unfermented apple juice, and "hard cider" indicates fermented products. This report uses the term "cider" in referring to fermented alcoholic beverages made from apples.

Cider is tax controlled by the United States Internal Revenue Code (IRC), which defines "hard cider" as "[Having] no more than 0.64g CO_2/100mL; derived primarily from apples/pears or apple/pear juice concentrate and water; containing no other fruit product or fruit flavoring other than apple/pear, and containing at least 0.5% and less than (not equal to) 8.5% alcohol by volume" 26 U.S.C. § 5041(b)(6) (Appendix A). Some cider and perry products paid at the Hard Cider tax rate may not be labeled "Hard Cider," "Perry," or "Cider" (TTB, 2018). Cider containing more than 7% Alcohol by Volume (ABV) must obtain a Certificate of Label Approval (COLA) from the TTB prior to bottling/producing. The IRC defines cider for taxation purposes, but a style guide was created by the American Cider Association that defines 14 different styles of cider, such

as Heritage Ciders, Modern Ciders, and Fruit Ciders. Each style is characterized by its aroma, flavor, appearance, and apple variety (American Cider Association, 2018). American Cider Association Cider Style Guidelines intend to clarify the various styles available to consumers.

The global cider market was valued at $10.7 billion in 2016 and is projected to reach $16.3 billion by 2023 (Statista.com 2018). The United States cider industry alone was valued at $1.3 billion in 2018 (Nielsen 2018). Craft and local cideries grew 30% in sales in 2016, while larger producers posted a slight decline in sales (Statista.com 2016). This trend shows how small cideries are becoming more popular compared to big brand companies. In the United States, a majority of cider producers are based in New York state, which has 93 recorded producers, followed by California and Michigan, each having 87 recorded producers (Nielsen 2017). With production rising, ensuring producers follow high safety standards is increasingly important.

Although the Alcohol and Tobacco Tax and Trade Bureau controls tax on cider, the FDA regulates cider's production and safety. Because cider contains alcohol, has a low pH, and been consumed for many years without illness, an assumption exists that cider production is safe. However published data validating this assumption is sorely lacking. Alcoholic beverage companies are required to comply with The Food and Drug Administration (FDA) Code of Federal Regulations Title 21 (CFR 21) Part 117, for example, Subpart B, Current Good Manufacturing Practices (cGMP). Included are cGMP's for sanitary operations, sanitary facilities and controls, processes, and other categories to ensure food safety. However, alcoholic beverage producers, including cider makers, are exempt from Subpart C, Hazard Analysis and Risk-Based Preventive

Controls (FSMA 2017, Ewing & Rasco, 2018). This means that cider producers are exempt from mandatory food safety plans, preventative controls, hazard analyses, and other measures (USFDA, 2015). Cider may be exempt from 21 CFR Part 117 Subpart C, but its production and final products differ from other fermented alcoholic beverages, such as beer and wine. For example, cider has a lower alcohol content compared to wine and does not contain hops like beer. While the risk of pathogens in cider may be considered low, apples—cider's main raw ingredient—are linked to numerous outbreaks related to foodborne pathogens. Therefore, investigating the growth of common pathogens during cider production is critical to protecting consumer health and safety.

The primary objectives of this study are the following:

1. Determine the survival or die off of *Escherichia coli* O157:H7, *Salmonella* spp., or *Listeria monocytogenes* during cider fermentation.

Hypothesis: *E. coli* O157:H7, *Salmonella,* and *L. monocytogenes* will not survive during the fermentation process.

2. Determine the bactericidal/bacteriostatic effects of pH and ethanol content on *Escherichia coli* O157:H7, *Salmonella* spp., or *Listeria monocytogenes*.

Hypothesis: With an increase in ABV and decrease in pH there will result in bactericidal effects on *E. coli* O157:H7

3. Compare the antibacterial effects of commercial hard ciders versus a model hard cider.

2. LITERATURE REVIEW

Cider producers must understand the potential risks associated with their products. Historically, cider was presumed safe due to its low pH and alcohol content. Because this assumption was not based on definitive studies, it does not ensure future safety. The following is a literature review covering cider production, apple-related outbreaks and associated foodborne pathogens, antimicrobial effects of ethanol and pH, and the fate of pathogens in fermented alcoholic beverages. The objective of this literature review is to clarify and understand the relationship between apple safety, cider production, and the antimicrobial effects of ethanol and pH, while highlighting the gaps in the literature with regards to ciders versus juice products.

2.1 Cider Production

2.1.1 Pre-Fermentation

The first step of cider making is selecting apple cultivars. Apple cultivars are classified and selected based on sugar, acid, and tannin levels. There are four apple cultivar classifications: sharp (containing high acid and low tannins); bittersharp (containing high acid and high tannins); bittersweet (containing low acid and high tannins); and sweet (containing low acid and low tannins) (Table 1). Cider production starts with raw apples, apple juice, or apple juice concentrate.

Table 1. Classification of Cider Apples (A. Lea, 2016).

Classification	Total Acid (%)	Tannin (%)
Sharp	> 0.45	< 0.2
Bittersharp	> 0.45	> 0.2
Bittersweet	< 0.45	> 0.2
Sweet	< 0.45	< 0.2

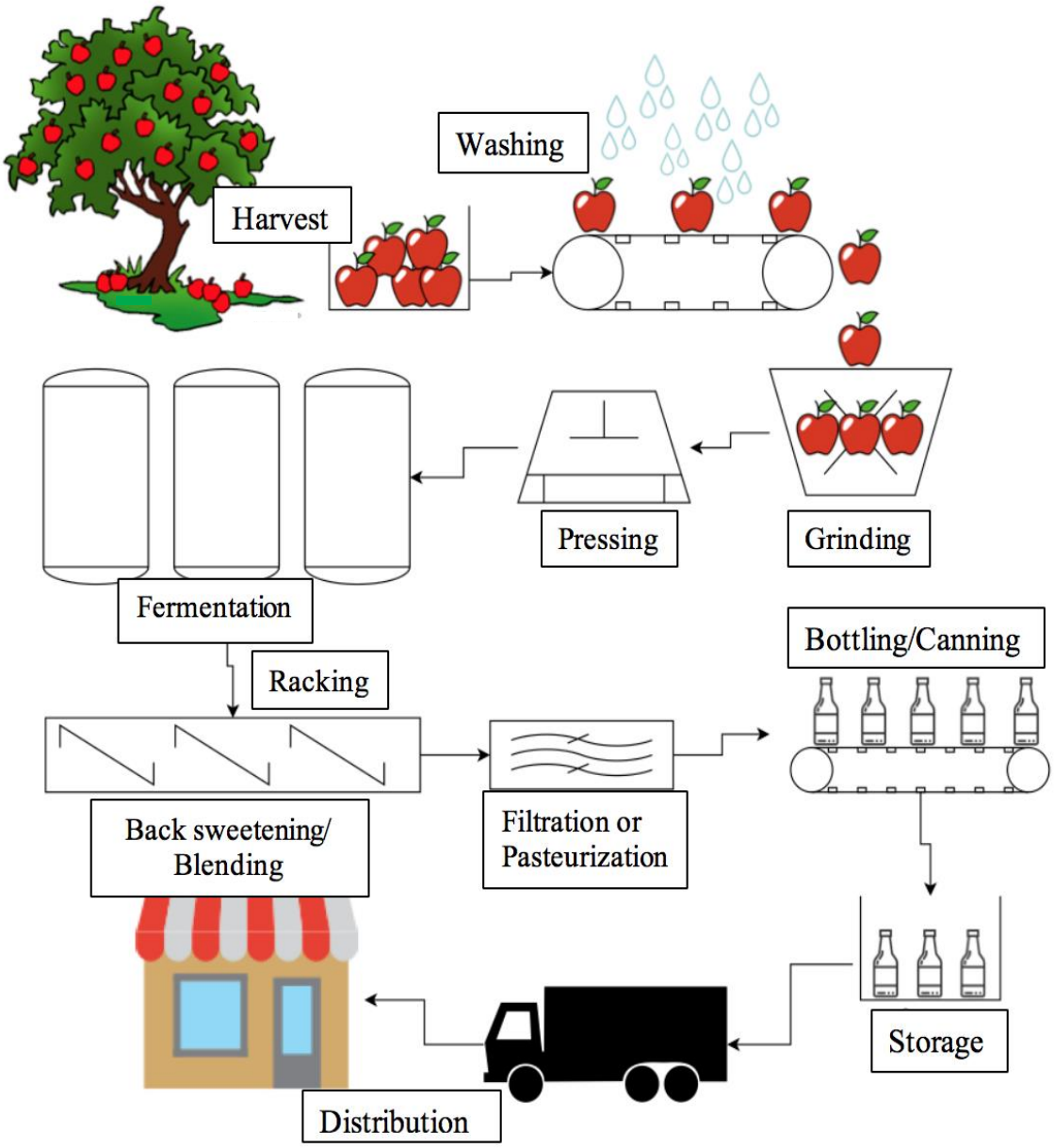

Figure 1. Process flow diagram for cider production

The overall scheme for cider production is shown in Figure 1. As noted the first step is the harvesting of apples. Apples are harvested by hand or mechanically when fully ripe. Consequently, they often are harvested from the ground (A. Lea, 2016). This process is potentially hazardous because the ground fosters unwanted microbes. If the skin of apples is punctured, bacteria can grow in the flesh (Ewing & Rasco, 2018). Once

harvested, apples sometimes undergo a process called “sweating”, in which apples are left in a controlled or uncontrolled warm dry place for one to two weeks to soften (Harte & Popa, 2002). At this stage, it is common for smaller producers to leave bins covered but in open areas without temperature control. Before milling, apples are washed to remove leaves, twigs, dirt, and bacteria (Gomes, Filho, Zielinski, Pietrowski, & Nogueira, 2014; A. Lea, 1995). Producers who press their apples, grind, crush, or mill them before pressing, which allows for optimum juice recovery. Producers who start with juice, generally use pasteurized juice. Before fermentation, soluble sugar (°Brix), pH, and titratable acidity are measured to ensure the final product meets the producer’s specifications. The initial Brix is important because it determines the final alcohol content of the cider. Acid and pH content are critical. If the acid content is low, then the pH may be too high, which means fermentation is susceptible to bacterial infections. If acid is high, then the final product’s flavor may be adversely affected (Jarvis, 2014). For the initial juice, the titratable acid in g/L malic acid should be around 0.3 – 0.7%, and the ideal pH ranges from 3.2 – 3.8. Traditionally used bittersweet apples tend to have high pH values and typically must be blended with other apple varieties to obtain a more acidic juice (Table 1). Adjustment to pH can also be done with the addition of malic acid. Another additive aiding fermentation is sulfur dioxide (SO_2), which helps inhibit the growth of spoilage yeast and bacteria, while still allowing desirable fermenting yeast to multiply. The dose is based on pH; in the lower pH range (3.0 – 3.3), less SO_2 is used (50 ppm), and at higher pH (3.3 – 3.8) more SO_2 is used (100 – 150 ppm) (A. Lea, 2016). Yeast nutrient is also added because of a lack of nutrients such as nitrogen and amino acids in apples. Yeast nutrient provides a blend of vitamins, minerals, amino acids, and

nitrogen compounds necessary for rapid, and complete fermentation and can be added to apple juice before fermentation, or at the beginning of the fermentation process (Rowell & Wagaman, 2015).

2.1.2 Fermentation

During fermentation, yeast converts simple sugars into alcohol and carbon dioxide. Typically, a yeast strain is selected and added to the juice based on the desired characteristics of the final product (Gutiérrez, Boekhout, Gojkovic, & Katz, 2018). For example, white wine and champagne yeast are often used in cider production. Another process used is wild fermentation, where yeast strains that are present in the environment initiate fermentation. Because there is a lack of control during wild fermentation, there are concerns for safety and quality. Fermentation is affected by pH, Brix, and temperature, which should be controlled. Yeast metabolism is greatly dependent on the temperature during fermentation, and variations of the fermentation temperature directly influence on the aromatic profile of the final cider (Cousin et al., 2017). Oxygen can also be a limiting element for yeast growth and can affect aroma production. The overall fermentation process for cider takes anywhere from two weeks to a few months (A. G. H. Lea, 1995).

2.1.3 Post-Fermentation

After fermentation, the primary concern becomes protecting the quality of the finished product from dead yeast and oxygen. Racking removes the cider from the solids that have formed at the bottom of the fermenting tanks, ensuring that cider is no longer in contact with any residual yeast. Residual yeast continues to metabolize and create off odors, which is a problem since aging is an important part of production. Cider is often

aged to smooth tannins and allow for full flavor development. After this step, producers have choices to maintain microbial stability: sulfite additions, pasteurization, or filtering (A. Lea, 2016). More sulfites can be added (up to the total legal limit of 200 ppm), which will kill off any residual yeast (Jolicoeur, 2011). Producers can pasteurize their product, but there is concern with affecting final product quality. Some producers choose to non-thermally process the product and filter to ensure microbial stability, although doing this can be expensive and may not be feasible for smaller cideries. Producers who choose not to apply any of these controls rely on the fermentation process and final product parameters, such as pH and alcohol content, to ensure the safety of their product. After fermentation, cider is carbonated, bottled, packed, and stored until distribution (Figure 1). There is insufficient literature published specifically looking at pathogen growth or survival in cider. There have been outbreaks associated with apples and apples juice, therefore understanding the risks associated with cider production is crucial. However, despite the presumed safety of ciders, risk for bacterial contamination may still exist. Previous findings show that *E. coli* O157:H7, *Salmonella spp*., and *L. monocytogenes* have cuased foodborne outbreaks in apple-based juices.

2.2 Apple and Apple Juice Safety

Bacterial pathogen presence on apples could be due to contamination from orchard soil, farm or processing equipment, harvesting, temperature abuse or physical abuse, such as the apple being bruised or wounded (Janes, Cobbs, Kooshesh, & Johnson, 2002). Because of these contamination risks, it is important to understand behavior of pathogens in these products to assess potential risks in cider. Studies have shown that pH and storage temperature play a role in *E. coli* O157:H7, *Salmonella*, and *L.*

monocytogenes survival and growth in apple juice and apples (Dingman, 1999; Fisher & Golden, 1998; González-López, Martínez-Peniche, Iturriaga, & Arvizu-Medrano, 2019; Salazar et al., 2016; Semanchek & Golden, 1996).

E. coli O157:H7 can survive in apple juice, therefore it's important to ensure the fermentation process can control pathogen growth. A study done by Semanchek & Golden (1996) investigated *E. coli* O157:H7 in apple juice stored at 20°C, after 10 days there was a 4 log CFU/ml decrease from the initial inoculum level of 6.5 log CFU/ml. The pH of the apple juice ranged from 3.62 initial to 3.72 final. Even with a decrease in population, *E. coli* O157:H7 still survived up to 10 days in the apple juice. Similarly, Dingman (1999) observed *E. coli* O157:H7 survival in apple juice with a pH of 3.8 at 22.5 and 4°C. From the initial inoculation level of 5 log CFU/ml, a 4 log CFU/ml decrease was observed at 22.5°C. At 4°C, at the same inoculation level, there was a 1 log CFU/ml decreased in 64 days. At refrigeration temperatures, the *E. coli* O157:H7 population remained stable. Both Semanchek and Golden (1996) and Dingman (1999) observed *E. coli* O157:H7 survival and how the pH and temperature can play a role. Although, it is important to look at other factors, such as alcohol content and time to help determine the survival of *E. coli* O157:H7 in cider.

Fisher and Golden (1998) observed the growth and survival of *E. coli* O157:H7 in Golden Delicious, Red Delicious, Rome, and Winesap apples which are common apples used for cider product. The populations were observed for up to 18 days at 4°C, 12 days at 10°C, and 5 days at 25°C. For this study, apples were cored, peeled, and stomached turning it into a slurry, then inoculated with 7 log CFU/mL *E. coli* O157:H7. After 5 days of storage at 25°C, the *E. coli* population decreased in Rome and Winesap apples but

increased by 1.0 log CFU/mL in Golden and Red Delicious apples. The pH of both the Golden and Red Delicious apples increased from 3.84 and 4.10 to 4.95 and 5.11, respectively, while the Rome and Winesap increased from 3.70 and 3.47 to 3.91 and 4.03, respectively. After 12 days of storage at 10°C and 18 days of storage at 4°C, all samples had a 1 log CFU/mL decrease. Growth was observed in Golden and Red delicious apples while the final pH was 4.95 and 5.11 and the starting pH's were higher than the Rome and Winesap apples (Fisher & Golden, 1998). In another study looking at ampicillin resistant *E. coli* O157:H7 in apple juice produced from Golden and Red delicious apples. The pH and Brix of the Golden and Red Delicious apple juices were 3.7 and 4.2, and 15.8°Brix and 14.0°Brix, respectively. The juice was inoculated with 5 log CFU/mL and there was no growth of *E. coli* observed in either of the juice samples but there was survival with just under 5 log CFU/mL present after 11 days (Dingman, 2000). *E. coli* can survive a wide range of pH's and it is concerning that *E. coli* O157:H7 survived in all apple juice and apple samples. Evidently the survival of *E. coli* O157:H7 effected by storage temperature and pH.

Not only is *E. coli* O157:H7 a concern, but *Salmonella* has also been associated with apples. A recent study shows that *Salmonella* can attach, colonize, and form biofilms on apple skin. To observe the fate of *Salmonella* on Golden and Red Delicious apples, the apples were wounded by with a small slice into the flesh and inoculated with 3 log CFU/apple and stored at 5, 15, and 22°C. At 22°C, *Salmonella* grew 3 log CFU/apple in 20 days, while Golden Delicious apples grew 2 log CFU/apple. At 15°C, *Salmonella* grew 2 log CFU/apple on both Red and Golden Delicious apples after 20 days. At 5°C, both of the apple varieties did not show an increase or decrease in

population, although *Salmonella* did survive the 20-day storage period (González-López et al., 2019). One limitation to this study was that the pH of the apples was not recorded. Others have reported the average pH of Red and Golden Delicious apples to be 3.8 and 3.6, respectively (Dingman, 2000). These results are similar to what was observed with *E. coli* O157:H7 in apples; in both Golden and Red Delicious apples, growth was observed. This could be due to the shared behaviors of Gram-negative bacterium, such as being more acid resistant than Gram-positive.

In a study looking at the survival of *L. monocytogenes* on fresh apples, the apples were inoculated with 6.9 log CFU/apple or 3.3 log CFU/apple on the stem end and skin of the apples, respectively. The apples were held at 5 and 25°C. The *L. monocytogenes* population recovered from apples after drying was approximately 2 log CFU lower than the initial inoculum. On the stem end of Gala apples stored at 5°C, at Day 0, there was 5.7 log CFU/apple recovered and by 15 days of storage, there was 5.5 log CFU/apple recovered, only decreasing the population by 0.2 log CFU/apple. On the surface of Gala apples stored at 5°C, at Day 0 there was 2.7 log CFU/apple recovered and by Day 15, there was no detectable *L. monocytogenes* measured. On the stem end of Gala apples stored at 25°C, at Day 0 there was 5.7 log CFU/apple recovered and by Day 15 there was 3.9 log CFU/apple still detectable. On the surface of Gala apples stored at 25°C, at Day 0 there was 2.7 log CFU/apple recovered, and by Day 15 there was no *L. monocytogenes* detectable. The skin of the apple was not able to foster the growth or survival of *L. monocytogenes* but on the stem end of the Gala apples was able to harbor the pathogen regardless of storage temperature (Salazar et al., 2016). Similarly, Gustafson and Ryser (2017) inoculated the top, middle, bottom, and core of Jonathan apples with *L.*

monocytogenes, a stick was inserted, and the apples were stored at 4 and 22°C. The apples had a pH range from 3.42 to 3.53. After 4 days of storage at 22°C, the population increased from 2.5 log CFU/mL to 5 log CFU/mL, while in 4°C the population took 14 days to increase from 2.5 log CFU/mL to 5 log CFU/mL in the apple core. At both 4 and 22°C, there was growth of *L. monocytogenes* on all parts of the apple.

In another study looking at *E. coli* O157:H7, *Salmonella* Enteriditis, and *L. monocytogenes* in apple juice, the juice was inoculated with 8 log CFU/ml of either three pathogens and stored at 5°C. With no added malic acid (pH 3.94), after 120 h of incubation, *E. coli* O157:H7 decreased by 1.5 log CFU/ml, *Salmonella* Enteriditis decreased by <1 log CFU/ml, and *L. monocytogenes* decreased by 1.1 log CFU/ml. At 5°C, apple juice supported the survival of all 3 pathogens for at least 5 days at refrigerated temperatures (Raybaudi-Massilia, Mosqueda-Melgar, & Martín-Belloso, 2009). And as explored in the previous section, there have been numerous outbreaks related to fresh fruit, and specifically apples and unpasteurized apple juice. The studies stated above show foodborne pathogens survival and growth in apple juice and apples. Apples and apple juice cannot inhibit the growth and survival of foodborne pathogens alone. With risk associated with apples and apple juice, fermentation or final product parameters such as ethanol content and the low pH of cider must ensure safety. As described, apple products are not resistant to bacterial contamination capable of causing outbreaks among consumers. Most studies have focused on apple juice and not cider.

2.3 Foodborne Pathogens and Apple-related Outbreaks

2.3.1 *Escherichia coli* O157:H7

Escherichia coli are Gram-negative, rod-shaped bacteria found in soil and water, and the microflora of the human gut (Table 2). Most *E. coli* strains are non-pathogenic although some strains, such as enterohemorrhagic *Escherichia coli* (EHEC) and have been associated with foodborne disease outbreaks. The *E. coli* serotype O157:H7 is the most prominent of the EHEC strains accounting for 75% of the EHEC infections around the world. The infective dose of *E. coli* O157:H7 can be as few as 10 – 100 cells. This toxin-mediated infection can cause hemorrhagic colitis resulting in severe cramps and abdominal pains, nausea or vomiting, fever, and bloody diarrhea. Hemorrhagic colitis can also lead to more serious diseases like hemolytic uremic syndrome (HUS) which can result in kidney failure (CDC 2019a).

Table 2 – Bacteria Characteristics

Pathogen	Gram +/-	Min pH	Max pH	Optimal pH range	Min Temp	Max Temp
Escherichia coli O157:H7	-	4.4	9.0	4.5 - 7.0	39°F	113°F
Salmonella spp.	-	3.7	9.5	6.5 - 7.5	41°F	115°F
Listeria monocytogenes	+	4.4	9.4	4.0 - 9.0	31°F	116°F

Pathogenic *E. coli* have been associated with foods such as raw or undercooked beef products, raw milk, and fresh produce. *E. coli* O157:H7 can develop an acid tolerance and has been found in acidic foods (<pH 4.6) such as products that have undergone lactic acid fermentation (i.e. yogurt, fermented sausages, and cheese) (Bad

Bug Book 2012). The CDC's Foodborne Outbreak Online Database reports that *E. coli* O157:H7 is the number one pathogen associated with raw apple cider (CDC 2016a).

According to the CDC's National Outbreak Reporting System (NORS), there have been 11 reported *E. coli* O157:H7 outbreaks since 1998 linked to unpasteurized apple juice ("National Outbreak Reporting System (NORS)," 2018). This does not include the recall that happened in 1996, where the Food and Drug Administration (FDA) recalled multiple Odwalla juice products, including its unpasteurized apple juice after a strain of *E. coli* O157:H7 was isolated from the juice. Officials did environmental sampling and testing in the juice but could not pinpoint the exact source. Odwalla's processing plant was known for violating many health and safety codes, having improper sanitation procedures, and not enforcing proper employee hygiene. The company was also accepting decaying apples from suppliers. More than 65 consumers were confirmed to be infected with *E. coli* O157:H7 contracted from Odwalla products, and the infestation caused the death of a child. More than a dozen of the 65 sickened, reported developing Hemolytic Uremic Syndrome (HUS), which is a life-threatening condition that can lead to organ failure. Because of this large outbreak, the FDA mandated that companies be required to put warning labels on unpasteurized juice products and to develop a Hazard Analysis Critical Control Point (HACCP) plans (63 FR 37030). In 1998 the FDA proposed Juice HACCP that required processors to achieve a 5-log reduction for the microbe identified as the most resistant microorganism of public health significance that is likely to occur in the juice. The final rule for juice HACCP was released in 2001, and became effective on January 22, 2002 (Anderson, 2001). There are exemptions for retailers or businesses that make and sell juice directly to consumers, but they must still

comply with FDA's food labeling regulation in 21 CFR 101.17(g) that requires a warning statement on packaged fruit juice products that have not been processed to prevent, reduce, or eliminate pathogenic microorganisms that may be present (21 CFR 120).

In 1999 in Oklahoma, while regulatory changes were progressing, 11 people became ill by *E. coli* O157:H7 from unpasteurized apple juice. Although the orchard that produced the apple juice was in compliance with the FDA, and had proper warning labels on their product, clearly the label insufficiently protects consumers and unpasteurized apple juice continues to cause illnesses (Diallo et al., 2011). Another apple juice company in Maryland, Baughers, was confirmed to be the source of an outbreak related to *E. coli* O157:H7 contamination in 2010. There were 16 reported individuals who contracted *E. coli* O157:H7, nine of which experienced bloody diarrhea, six required hospitalization, and three developed HUS. Of the 16, 12 of them were children who were between the ages of 2 – 13 years old (Marler Clark 2010). In 2016, there was an *E. coli* O157:H7 outbreak at the Louisburg Cider Mill Festival in Kansas. There were 56 reported illnesses, 10 of which were hospitalized, and two developed HUS. The source of contamination was unpasteurized apple juice. It is clear that *E. coli* contamination is a risk in unpasteurized apple juice, and could be a risk for the cider industry (Besser, 1993).

2.3.2 *Salmonella* species (spp.)

Salmonella is a Gram-negative, non-spore forming, facultative anaerobic bacterium that can be found in water and soil contaminated with fecal matter (Table 2). The species *Salmonella enterica* is the greatest public health concern and the most common species associated with human illness in the United States. *S. enterica* can cause two types of illness depending on the serotype; nontyphoidal salmonellosis and typhoid

fever caused by *S.* Typhi and *S.* Paratyphi A. The infective dose of non-typhoidal salmonellosis, which is the disease most frequently associated with foodborne illness, can be as low as one bacterium. Symptoms include nausea, vomiting, abdominal cramps, diarrhea, fever and headache (Food and Drug Administration, 2012).

Salmonella is commonly found in egg, poultry, and dairy products, fresh produce, and can also be found in the environment. The CDC estimates that *Salmonella* in food causes approximately one million illnesses leading to 19,000 hospitalizations and 380 deaths in the United States each year (CDC 2019). Since 1999, there have been two reported *Salmonella* outbreaks related to apple cider ("National Outbreak Reporting System (NORS)," 2018). Although *Salmonella* outbreaks in apple products are not as prevalent as *E. coli* O157:H7, it is a major concern for the fresh produce industry due to its small infective dose.

2.3.3 *Listeria monocytogenes*

Listeria monocytogenes is a Gram-positive, rod-shaped, facultative bacterium (Table 2). The CDC (2016) estimates that *L. monocytogenes* causes about 1,600 illnesses each year in the United States with more than 1,500 hospitalizations and 260 deaths. *L. monocytogenes* is ubiquitous in the environment, soil, and decaying vegetation. The infective dose varies with serotype, food type, and susceptibility of the host. In some cases, fewer than 1,000 bacteria may induce symptoms. Pregnant women, fetuses, infants, immunocompromised, and elderly are more susceptible to infection. There are two forms of disease that *L. monocytogenes* can cause in humans; non-invasive gastrointestinal illness and the invasive form which can lead to listeriosis (Food and Drug Administration, 2012). Non-invasive gastrointestinal illness symptoms include fever,

muscle aches, nausea and vomiting and over time individuals may develop listeriosis. Symptoms of this include inflammation of vital organs (septicemia) or protective membranes covering the brain and spinal cord (meningitis) and spontaneous abortions in pregnant women.

L. monocytogenes is resistant to low pH, high salt, and low temperatures and has been linked to outbreaks in fruit and vegetable juices, fresh produce, but not known to specifically affect apples until 2015 (Gustafson & Ryser, 2017). The CDC reports between 1998 – 2017 there were two listeriosis outbreaks associated with apples. In January 2015 a total of 35 people were infected with *L. monocytogenes* linked to the consumption of caramel apples resulting in 34 hospitalizations and seven deaths. People who were severely infected experienced listeriosis, fetal loss, and meningitis which lead to some of the reported deaths (CDC 2015). The contaminated caramel apples produced were utilizing Bidart Bros. Granny Smith and Gala apples. *L. monocytogenes* was traced to Bidart Bros. packing facility in Bakersfield, California. This raised concern for *Listeria* on and in apple products. *L. monocytogenes* was not previously considered a threat to the apple industry and because of this there was a lack of literature available on *L monocytogenes* on fresh apples. Two studies were published after the outbreak in 2015. One of which observed the fate of *L. monocytogenes* in fresh apples and caramel apples and another studied *L. monocytogenes* on fresh apples at various temperatures (Salazar et al., 2016; Sheng, Edwards, Tsai, Hanrahan, & Zhu, 2017). It was unknown how the caramel apples of the outbreak became the vector for listeriosis because apples have undesirable characteristics, such as low pH and tough skin, that do not allow for the growth of *L. monocytogenes.* Due to the lack of research and the caramel apple outbreak,

more information was needed on the survival of *L. monocytogenes* in apples (J. H. Ryu & Beuchat, 1998). There is significant data that suggests apple processing and cider production can result in bacterial contamination risks to the consumer.

2.4 Fermented Alcoholic Beverage Safety

2.4.1 Wine

Articles have been published on pathogens in other fermented alcoholic beverages such as wine and beer that may help predict the fate of foodborne pathogens in cider. For example, wine contains many antimicrobial parameters including high ethanol content, sulfites, polyphenols, low pH, malic and tartaric acid. A study done by Møretrø & Daeschel (2006) looking at the efficacy of wine against foodborne pathogens, found that wine was strongly effective against *S.* Typhimurium*, E. coli* O157:H7, and *L. monocytogenes*. Chardonnay white wine and Cabernet Sauvignon red wine were inoculated with 7.0 log CFU/mL of either *E. coli* O157:H7, *S.* Typhimurium, or *L. monocytogenes.* The ABV of the white wine was 11.9%, while the red was 12.6%, with both samples having a pH of 3.5. The wines also had varying levels of tartaric acid, red with 7.3 g/L and white with 6.4 g/L. In red wine, *E. coli* O157:H7 reach a > 6.0 log CFU/mL decrease to undetectable levels in 30 min and *S.* Typhimurium became undetectable after 10 min. For *L. monocytogenes* it took 60 mins to become undetectable with a > 6.0 log CFU/mL reduction. *E. coli* O157:H7 and *L. monocytogenes* reached a 6 log CFU/mL reduction, but still was detectable after 30 and 60 min, respectively. In all cases, complete die off was observed quicker in the red wine, than in the white wine. This result may be due to red wine having a higher alcohol content and lower titratable acidity (Møretrø & Daeschel, 2006). The average alcohol content of wine is 7 – 13%, which is

higher than that of beer and cider (Kerr & Stockwell, 2012). With characteristics different than cider, such as higher alcohol content and more sulfites and polyphenols, pathogens are not expected to survive the environment of wine.

2.4.2 Beer

Beer has intrinsic and extrinsic antimicrobial hurdles helping to ensure the quality and safety of products. Extrinsic factors include certain processing techniques such as mashing, boiling of wort, pasteurization, and filtration. Pathogens are likely inactivated by processes, such as the use of heating. Intrinsic factors include ethanol, hop bittering compounds, low pH, high carbon dioxide, low oxygen, and lack of nutrient substances. Hops are important bitterness and aromatic flavor compounds in beer and have natural antimicrobial effects. Hops help prohibit growth and limit survival of Gram-positive pathogens such as *L. monocytogenes* (Menz et al., 2011). The mechanism in which hops inhibit spoilage bacteria in beer is due to the acids present in hop resins, such as iso-α-acids, which incorporate into cell membranes, penetrate bacterial membranes, and change the intracellular pH, which lead to loss of cell function (Gutiérrez-Larraínzar et al., 2012; Karabín, Hudcová, Jelínek, & Dostálek, 2016).

In a study of the efficacy of beer on foodborne pathogens, beer was inoculated with *E. coli* O157:H7, *Salmonella,* and *L. monocytogenes* and stored at various temperatures to observe growth and survival of the pathogens. Three different beers were inoculated with pathogens. The pH of the beer was 4.3, 4.3, 4.2, and the ABV was 5.0, 4.6, 5.0% respectively. The three beers were inoculated at 3.5 log CFU/mL of *L. monocytogenes* and stored at 5 and 22°C. When stored at 5°C, bacteria died off after 3 days, and at 22°C, *L. monocytogenes* died-off after 1 day in all three samples. Beer was

inoculated with 3.5 log CFU/mL of *E. coli* O157:H7 and stored at 5 and 22°C. The die off of *E. coli* O157:H7 took 14 days at 22°C, but only reached a 1.6 log CFU/mL reduction at 5°C during a 28-day incubation period. When inoculated with 3.5 log CFU/ml *Salmonella* Typhimurium stored at 5°C, there was a decrease by 2.3 log CFU/ml in 26 days at 5°C. At 22°C *Salmonella* died off after 7 days of incubation (Kim, Kim, Lee, Hwang, & Rhee, 2014).

Aldred et al. (2011) observed survival of *E. coli* O157:H7 in 5% beer with a pH of 4.3 and a hop level of 12 IBU/ppm showed to reduce the bacterial population to 2 log CFU/mL in 10 days. With an initial inoculum level of 3 log CFU/mL was used, the pathogens could survive for 10 days in the 5% beer being stored at 4°C. However, both *Salmonella* and *E. coli* were unable to grow in the beer. Similarly, to Hwang et al. (2014), *E. coli* O157:H7 was able to survive in beer with 5% ABV in refrigerated temperatures. Hops are known for being effective against Gram-positive bacteria, and as the data shows, *L. monocytogenes* in beer was effectively killed off by 1 day while *Salmonella* and *E. coli* survived longer. A previous study showed an environment with 80 IBU, iso-α-acids did not affect the growth of *E. coli* O157:H7 and *Salmonella* Typhimurium. However, both *Salmonella* and *E. coli* were unable to grow in the beer (Menz et al., 2011). These studies show that there is a possibility of pathogen survival in beer, even with hurdles such as ethanol, hops, and low pH.

2.4.3 Cider

Minimal literature exists about foodborne pathogens in cider. Historically cider has been deemed safe due to characteristics such as ethanol content, low pH, and sulfite content, which with the presence of organic acids, being effective against pathogen

growth and survival. In order to investigate the fate of pathogens in cider, Semanchek & Golden (1996) inoculated apple juice with 6.4 log CFU/mL of *E. coli* O157:H7 and stored at 20°C. After 3 days of fermentation, the ethanol content was 3.03%, with a 3 log CFU/mL reduction of *E. coli* O157:H7. After 10 days the cider had a 6 log CFU/mL reduction and the bacterium was undetectable. The final ABV of the cider was 6%. The pH of the cider did not change significantly ($p > 0.05$), ranging from 3.62 initial to 3.75 final. Ultimately this study had some limitations in that it is the only published literature in cider safety and researchers did not observed *Salmonella* or *L. monocytogenes* and only used one strain of *E. coli* O157:H7 that was not acid adapted. In the cider industry, a gap exists in this area of research and further research should be done on a wider range of ethanol contents, pH, and organisms. Further investigation is needed to understand pathogen behavior in cider. Articles have been published on pathogens in other fermented alcoholic beverages such as wine and beer that may help predict the fate of foodborne pathogens in cider.

2.5 Mechanism of The Anti-bacterial of Fermented Alcoholic Beverages

2.5.1 Overview of Bacterial Structure and Physiology

Bacteria are classified as being Gram-positive or Gram-negative based on their peptidoglycan structure in the cell wall (Table 2). Cross-linking of amino acids in the peptidoglycan helps maintain the shape and strength of the cell, while protecting from osmotic lysis (Coleman & Smith, 2014). The cell wall is a strong, flexible structure composed of peptidoglycan, which consists of sugars and amino acids cross-linking to form a strong mesh-like structure (Figure 2). The plasma membrane is a layer made of

phospholipids and proteins, regulating the flow of materials in and out of the cell, while engaging in interactions with the environment (Wicken, 1985).

Much like bacterial cells, yeast cells have a cell wall, plasma membrane, and cytoplasm with other cell structures. However, the cell walls in fungal cells are polysaccharides and glycoproteins, consisting of glucan, chitin, and mannoproteins (Salazar Monroy, 2016). Fungal cells are known for having a resilient cell wall, which is associated with cross-linking between different components of the cell wall (Figure 3).

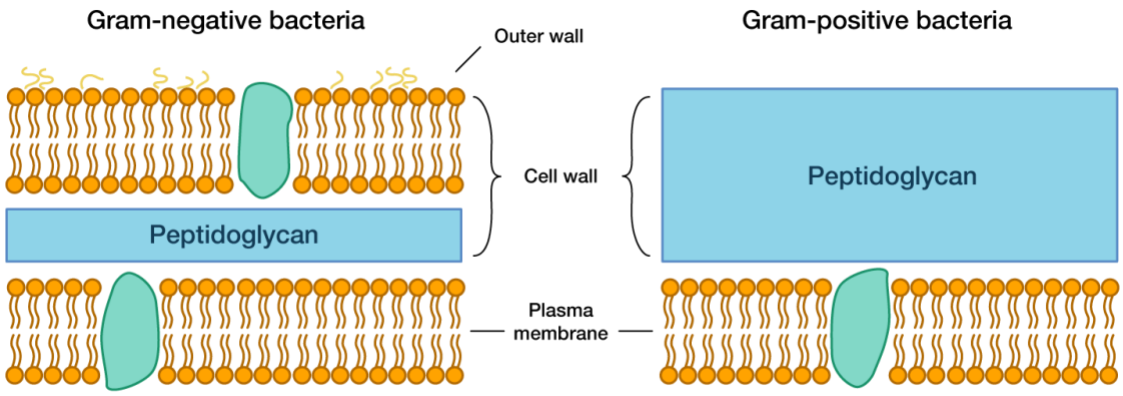

Figure 2. Cell wall structure of Gram-positive and Gram-negative bacteria (Adapted from Online Biology Notes. 2017. Bacterial Cell wall: Structure, Composition and Types)

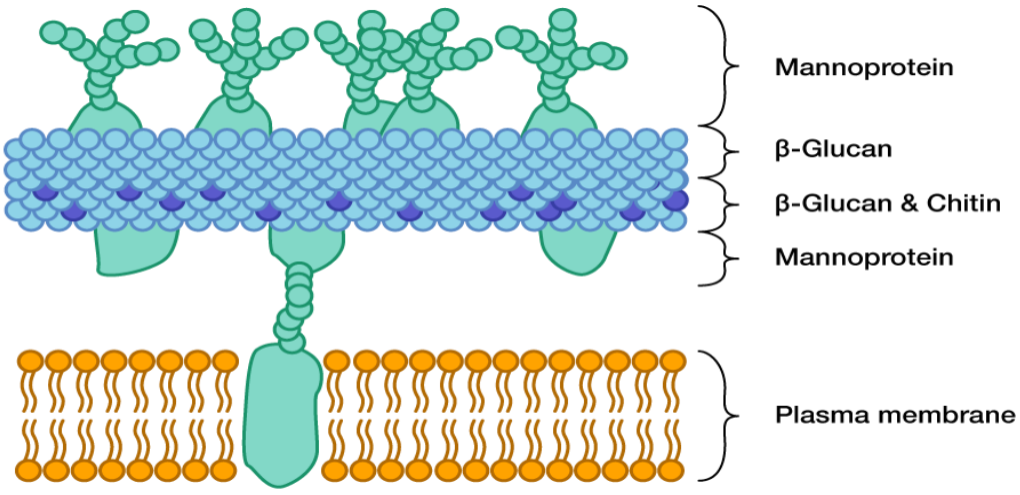

Figure 3. Cell wall structure of fungal (yeast) cell (Adapted from McClanahan, 2009)

2.5.2 Effect of Ethanol on Cell Wall and Metabolism

There have been no outbreaks in legally produced fermented alcoholic beverages, likely due to the presence of ethanol. In the food and beverage industry, low concentrations of ethanol function as a food preservative and is bacteriostatic at low concentrations. However, higher concentrations of ethanol are needed to inhibit yeast cells. Ethanol can disrupt cell wall structure, resulting in a decrease in membrane permeability leading to an overall loss of cell function. Bacterial cells consist of a cell wall, plasma membrane, and the cytoplasm containing cell structures such as ribosomes, a chromosome, and plasmids. The antimicrobial mechanism of ethanol acts primarily on the cell wall and plasma membrane.

Ethanol results in peptidoglycan disarrangement altering its function. This is done by direct protein interactions, such as protein-alcohol binding sites, although little is understood about this mechanism (Ingólfsson & Andersen, 2011). Gram-positive bacteria are highly cross-linked with peptide bridges, creating a thick peptidoglycan cell wall with strong cross-linking (Man, Gâz, Mare, & Berţa, 2017). Gram-negative bacteria are partially cross-linked with a thin peptidoglycan wall, creating a weaker structure, making them more susceptible to ethanol.

Some yeast species, specifically *Saccharomyces cerevisiae*, show tolerance and can adapt to high concentrations of ethanol. Studies have documented the alteration of cellular lipid composition in response to ethanol exposure (You, Rosenfield, & Knipple, 2003). This alteration allows the yeast to survive an environment with ethanol present because it generally dies off after metabolizing sugars, producing alcohol, and CO_2 during fermentation.

Weakened hydrophobic associations also affect enzymes present that help cross-link peptidoglycan. Weak cross-linking decreases the strength of the cell structure leading to overall weakened function. Ethanol also affects cell division by altering the membrane association of proteins that function in peptidoglycan synthesis (Ingram 1981). Adding ethanol to an aqueous solution weakens hydrophobic associations, increases membrane permeability, and destabilizes proteins. Bacteria also contain cell membranes, which consist of phospholipids and proteins. It has long been known that alcohols alter lipid bilayer properties and membrane protein function, although challenges remain in analyzing the underlying mechanism of alcohol-induced changes in membrane protein function and whether the cause is from direct alcohol-protein interactions or from changes in lipid bilayer physicochemical properties. It is proven that ethanol reduces bilayer stability breaking down barrier properties, causing increased ion permeability (Ingolfsson et al. 2011). Increasing the permeability of the membrane allows more protons to pass into the cytoplasm, decreasing the ability of the cell to maintain pH homeostasis, and resistance to stress (Barker et al. 2001). This disruption interferes with the distribution of solutes within the cytoplasm, leading to the sensitization of the cell to osmotic stress. Once the cell is under stress, the indirect effects include an inability to uptake nutrients or multiply, which eventually lead to cell death (Lonnie O. Ingram, 1989).

The mechanism associated with ethanol stress of yeast cells is poorly understood. However, research shows that yeast subjected to ethanol stress struggle to maintain energy production, leading to an increased expression of genes associated with energy-generating activities such as glycolysis and mitochondrial function. With such disruptions

occurring to the cell, growth is inhibited (Stanley, Bandara, Fraser, Chambers, & Stanley, 2010).

To determine the bactericidal effects of ethanol, it is necessary to observe the relationship between concentration and time. For example, if a finished cider product was contaminated during bottling, it is important to note the time needed for a pathogen to die off, if at all, at a specific ethanol concentration. There has been minimal research done on alcohol's antibacterial capabilities, which may be due to a lack of foodborne pathogen related outbreaks in alcoholic beverages. The use of ethanol alone may only be effective at killing bacteria at higher levels than current levels found in most commercial ciders on the market, which range from 1 – 7% ABV (Kosseva, Joshi, & Panesar, 2017). Also, worth studying is the sensitization of ethanol on various pathogens of concern.

Because *E. coli* O157:H7 is a Gram-negative bacterium, it is more susceptible to an ethanol induced environment (L. O. Ingram, Vreeland, & Eaton, 1980). A study done in "alcohol-free" beer (pH 4.3) as a base medium looked at the effect of ethanol on *E. coli* O157:H7 (initial load of 3 log CFU/ml). "Alcohol free" beer with an initial ethanol content of 0.5% was adjusted to various ethanol concentrations (2.7 and 5%). At 0.5% ethanol, the pathogen grew by 4 logs; at 2.7% ethanol there was a 3-log reduction in 40 days; at 5% ethanol there was a 3-log reduction in 30 days. Therefore, it is possible for *E. coli* O157:H7 to survive up to 40 days at 0.5 and 2.7% ethanol, even in the presence of hops, which are known for having antimicrobial properties (Menz, Aldred, & Vriesekoop, 2011). Another study looked at *E. coli* O157:H7 (1.0×10^6 CFU/ml) in phosphate-buffered saline (PBS) containing two different ethanol concentrations (2 and 5% [vol/vol]). Samples were stored at 18 and 30°C and sampled at 4 h and 1, 3, 5, and 9

days. In the sample containing 5% ethanol at 30°C, the bacteria died off after 9 days (> 6.0 log reduction). At 5% ethanol at 18°C, the population decreased by 2 log CFU/ml in 9 days. In the samples containing 2% ethanol stored at both 18 and 30°C, there was no decrease in population and the bacteria survived (Masuda, Hara-Kudo, & Kumagai, 2016). After a 9-day storage period, bacteria were still detected, presenting a potential hazard.

Salmonella spp. is another Gram-negative foodborne pathogen capable of surviving and adapting to harsher environments. A study of *Salmonella enterica* serovar Enteritidis, evaluated its fate in various ethanol concentrations. Preliminary studies show that *Salmonella* was able to grow in LB broth with a pH of 7.0 containing 0, 2.5, and 5% ethanol. It is important to note that this *Salmonella* was not ethanol adapted prior to inoculation into an ethanol induced environment, therefore it is concerning that the bacterium grew in the presence of ethanol. *Salmonella* was ethanol adapted in Luria-Bertani (LB) broth supplemented with 2.5, 5.0, 7.5 and 10% ethanol. *Salmonella* was inoculated at 1 x 10_6 CFU/mL into a 15% ethanol solution. In the sample containing 2.5% ethanol adapted *Salmonella*, there was less than 1 log CFU/ml survival, and in the sample with 5%, 7.5%, and 10% ethanol adapted cells, there was a 1 log decrease after 1 h. This study shows that ethanol adapted *Salmonella* spp. could survive potentially lethal levels of ethanol while still maintaining membrane integrity (He, Zhou, Shi, & Shi, 2016).

Sensitization of *Listeria monocytogenes* is dependent on ethanol concentration (Barker & Park, 2001). A study looked at *L. monocytogenes* in TSBYE (pH 7.0) with varying ethanol concentrations (0.63, 1.3, 2.5, 5.0% ethanol) stored at 35°C for 24 h. At

0.63% ethanol *L. monocytogenes* grew 4 log CFU/ml in 12 h. At 1.3% ethanol, the pathogen grew 4 log CFU/ml in 16 h. At 2.5% ethanol, it grew 4 logs in 18 h. In the presence of 5% ethanol, the population grew by 1 log in 24 h (Oh & Marshall, 1993). Barker and Park (2001) observed 0, 1.25, 2.5, 5.0, and 10% ethanol at pH 7.0 and there was no significant loss in viability after 90 mins. *L. monocytogenes* was able to grow in solution with up to 5% ethanol present, and even survive in the presence of 10% ethanol. As stated prior, Gram-positive bacterium are more ethanol resistant than Gram-negative and observations are as expected. Importantly, ethanol is not the only parameter contributing to cider safety, as pH of the product also may play a major role.

2.5.3 Effect of pH on Cell wall and Metabolism

Microorganisms can grow in a wide range of pH's depending on the type and strain of organisms and the food or growth medium. Every microorganism has a minimum, optimum, and maximum pH for growth (Table 2). Acidity can be measured in two ways, through potential hydrogen (pH) and/or total acidity or titratable acidity (TA). The pH is the measure of protons (H+) in a solution, while titratable acidity is the measure of the sum of organic acids presence in a product (Bjornsdottir, Jr, Mcfeeters, & Breidt, 2006; Jolicoeur, 2011).

The mechanism in which bacteria can be killed by acids and low pH is by undissociated organic acids dissociating in solution and cause structural damage to the cell membrane, DNA, and proteins (Bjornsdottir et al., 2006; Timbermont et al., 2006). Once the cell structure is damaged, protons can easily move in and out of the cell, decreasing cytoplasmic pH, causing the cell to lose function. Change of pH in the intracellular cytoplasm also affect the proteins found in the cell membrane of the bacteria,

causing damage to the cell by weakening the cell wall and increasing membrane permeability, which will affect the cells ability to regulate proton flow (Booth, 2003).

Organic acids are used as a preservative, because they can decrease the pH of foods and beverages. Studies have been conducted evaluating the inhibitory effects of organic acids on various foodborne pathogens. The mechanism related to organic acids inhibitory effect is linked to its undissociated form which is related to pKa (Bjornsdottir et al., 2006). Weak acids will not completely dissociate in water, meaning the acid is left undissociated. The amount of dissociation determines pH. Weak acids—such as propionic > acetic > malic > citric > lactic > tartaric acids (listed in order of pKa values)—in their undissociated form are able to pass through cell membranes, preventing bacterial growth (Wang et al., 2018). This is done by the weak acid dissociating, releasing protons, and acidifying the cytoplasm (Cotter & Hill, 2003). The type of acid that is present in the environment is important for inhibition. Some organic acids are more effective than others, depending on the ratio of the dissociated to undissociated compounds present in solution, and pH level.

As discussed in section 2.4.1., Gram-negative and Gram-positive bacteria have differing cell structure and are affected by change in pH and the presence of organic acids. Gram-negative bacteria have an outer membrane that is naturally hydrophobic, helping to block the entry of hydrophilic molecules, including monosaccharides, amino acids, and nucleosides. By contrast, Gram-positive bacteria have a thick peptidoglycan layer and lipid bilayer in their cell membrane (Raybaudi-Massilia et al., 2009). Gram-positive bacteria have no outer wall, which means organic acids easily enter cells, making their intrinsic resistance is relatively low. The effectiveness of organic acids will change

depending on if the bacterium is Gram-positive or Gram-negative. This would help explain the sensitivity of *Listeria monocytogenes* sensitivity to malic acid compared to *E. coli* O157:H7 and *Salmonella Enteriditis*, which will be further discussed in a future section.

Malic acid is the predominant organic acid found in apples and can affect the characteristics of final cider products. For cider products, acidity and pH play a critical role. Most apple juices used for cider have a pH ranging from 3.2 – 3.8 to ensure desired organoleptic properties of their final product. The target titratable acidity for cider makers is 4.5 – 7.5 g/L (0.5% - 0.8%) malic acid (Jolicoeur, 2011). Generally, the malic acid percent in unprocessed apple juice is between 0.3 – 0.6%, although some variety of cider apples can have up to 0.7%, and sometimes cider makers will add malic acid to achieve their ideal titratable acidity. (A. Lea, 2016).

Malic acid in various juices have a different effect on pathogens. Apple, pear, and melon juice were inoculated with 8 log CFU/mL of *E. coli* O157:H7, *Salmonella* Enteritidis, or *L. monocytogenes* and various concentrations of malic acid. These organisms were not acid adapted, and all samples were stored at 20°C. The pH values for apple juice at 0.4, 0.6, and 0.8% malic acid was 3.3, 3.1, and 3.1, respectively. For pear juice with 0.4, 0.6, and 0.8% malic acid, the pH's were 3.5, 3.3, and 3.2, respectively. The pH's for melon juice with 0.4, 0.6, and 0.8% malic acid were, 3.8, 3.6, and 3.5, respectively. The higher percent malic acid (0.8%) in apple juice at pH 3.1 was able to kill off all three pathogens. When the pH was increased to 3.2 in the pear juice at the same malic acid concentration, *E. coli* O157:H7 was the only one able to survive, having a 1.7 log reduction from the original 8 log CFU/ml inoculation level. And in melon juice

with a pH of 3.5 with a 0.8% malic acid concentration, *E. coli* also survived a 1.8 log reduction (Table 3). As stated in section 1.5.3, Gram-positive bacterium is less acid resistant than Gram-negative. As expected, there was more die-off observed in *L. monocytogenes* at all levels of malic acid.

Table 3. Effect of malic acid and time on pathogens in apple, pear, and melon juice (Raybaudi-Massilia et al., 2009).

Organism	Storage Time (h)	Malic Acid (%)	Survival population in Juice (log CFU/ml)*					
			Apple	pH	Pear	pH	Melon	pH
E. coli O157:H7	0	0.4	6.6	3.3	6.4	3.5	6.6	3.8
		0.6	6.7	3.1	6.4	3.3	6.5	3.6
		0.8	6.7	3.1	6.4	3.2	6.5	3.5
	24	0.4	6.6	3.3	6.1	3.5	6.5	3.8
		0.6	3.2	3.1	6.3	3.3	6.5	3.6
		0.8	<1	3.1	6.3	3.2	6.2	3.5
Salmonella Enteriditis	0	0.4	7.5	3.3	6.0	3.5	6.0	3.8
		0.6	7.4	3.1	6.0	3.3	6.1	3.6
		0.8	7.2	3.1	5.8	3.2	5.9	3.5
	24	0.4	5.3	3.3	5.2	3.5	5.3	3.8
		0.6	2.9	3.1	3.4	3.3	3.7	3.6
		0.8	<1	3.1	<1	3.2	<1	3.5
Listeria monocytogenes	0	0.4	7.0	3.3	6.6	3.5	6.5	3.8
		0.6	6.7	3.1	6.4	3.3	6.3	3.6
		0.8	3.4	3.1	5.8	3.2	6.2	3.5
	24	0.4	<1	3.3	2.1	3.5	6.3	3.8
		0.6	<1	3.1	<1	3.3	5.3	3.6
		0.8	<1	3.1	<1	3.2	<1	3.5

*Initial load of 8 log CFU/ml

Importantly, the pHs observed in this study are lower than that found in cider. As stated prior, the ideal pH range for cider is 3.2 – 3.8, therefore if there is survival in apple juice with a pH as low as 3.1 this is a concern for cider production. Since *E. coli* O157:H7, *Listeria monocytogenes,* and *Salmonella* spp. can adapt to acidic environments, apple juice alone cannot kill pathogens, therefore the effectiveness of low pH, organic acids and ethanol in combination is necessary.

Many factors such as final pH, type of acid present, and a bacterium's ability to acid adapt will have an effect on the growth or survival of pathogens. *E. coli* O157:H7 can adapt to acidic environments when grown in acidic media, making possible its survival in a wide range of systems (Miller & Kaspar, 1994; Raybaudi-Massilia et al., 2009). When acid adapting in tryptic soy broth with glucose (TSBG), the organisms ferment the glucose and produce acid, lowering the pH. This fermentation and acid production occurs slowly, becoming acid adapted and not acid shocked (Buchanan & Edelson, 1996). In a study done by Beuchat et al. (1998), *E. coli* O157:H7 cells were acid adapted in TSBG and plated on TSA acidified with malic, citric, lactic or acetic acid. After 48 h incubation, acid adapted *E. coli* O157:H7 plated on TSA acidified with malic and citric acid and a pH of 4.2 and 3.9, both samples had populations of $10^2 – 10^3$ CFU/mL. At the same pH, when acetic acid was used as an acidulant, there was no survival at pH 3.9 or 4.2. This means that the inhibition by acetic acid is more effective than malic. In this study, malic and citric acid were least effective in killing off *E. coli* O157:H7. They were not lethal to *E. coli* O157:H7 at pH's ≥4.5 and their optimal pH range for growth is 4.5 – 7 (Table 2). The type of acid that is used to acid adapt organisms, affect the behavior of the bacteria (J. Ryu, Deng, & Beuchat, 1999).

As shown in Table 2, *Salmonella'*s optimal pH range for growth is 6.5 – 7.5, but can survive in acidic foods at lower pH values for long periods of time (Álvarez-Ordóñez et al., 2013). In a study done by Nogueira et al. (2003), acid-adapted *Salmonella* was inoculated into apple (pH 3.7), orange (3.7), pineapple (3.6), and white grape (3.6) juice concentrates at 3 or 4 log CFU/mL. The juice concentrates were stored at -23°C. At 12 weeks a 2 log CFU/mL reduction was observed but *Salmonella* was still detectable at 12

weeks. *Salmonella* was never eliminated during storage in any of the samples. Looking at only freezing temperatures is a disadvantage knowing that the temperature range for *Salmonella* growth is 5 – 45°C. If the study were to look at higher temperatures, even close to refrigeration there is opportunity to observe bacterial growth or survival. Leyer and Eric (1992) observed a 7 log CFU/mL die-off of *S.* Typhimurium in electroporation buffer acidified to pH 3.85 with lactic acid in 1 h but recovered bacterium in the buffer acidified with acetic acid in 2 h. Although when the *S.* Typhimurium was acid adapted the bacterium was able to survive up to 60 mins in the lactic acid solution and 2 h in the acetic acid solution. Lactic acid is a weaker acid than acetic acid, therefore it is interesting that the study recovered *Salmonella* in the acetic acid solution. Another study comparing acetic, lactic, citric, and tartaric acid has reported that lactic acid has the strongest inhibitory affect on *S.* Typhimurium (Baaboua et al., 2018). Conclusions on why lactic acid is a stronger inhibitor than acetic may be due to certain gene expression but is still unclear. Other research has indicated that in orange juice with a pH of 3.8 stored at 0°C, the population of *S. enterica* serovar Typhimurium decreased to undetectable levels (<2 MPN/mL) from an initial population of 6 log CFU/mL. The organic acid present in orange juice is citric, which has a pKa of 3.1. Nevertheless, storage temperature and type of acid present is likely critical for the survival of bacteria in juices, concentrates, and acidic environments (Nogueira, Oyarzabal, & Gombas, 2003). But malic acid is the most relevant to this study.

L. monocytogenes is a Gram-positive bacterium and is expected to be less acid resistant than Gram-negative bacterium as explained in section 2.5.2. To observe the effect of pH on *L. monocytogenes,* the bacteria was inoculated into a solution created

with 50 mM of DL-Malate to achieve a pH of 3.0. The malic acid solution was effective at reducing *L. monocytogenes* by a 4 log CFU/mL in 6 min. Although, when the pH was raised to 4.0 (15 mM DL-Malate), *L. monocytogenes* only reduced by 2 log CFU/mL in 120 min (Barker & Park, 2001). The pKa of malic acid is 3.4 meaning at pH 3.4, 50% of the acid is dissociated and the other 50% is undissociated. At a higher pH like 4.0, there is less dissociation of the acid and will be less effective causing cell damage. *L. monocytogenes* can grow at a minimum pH of 4.5, but as the study shows can survive in pH 4.0. The addition of malic acid overall aids in lowering pH but is dependent on quantity that is present or added and the *L. monocytogenes* in this study was not acid adapted.

2.6 Synergistic Effects of Ethanol and pH

Ethanol and pH work synergistically to inhibit pathogen survival or growth (Jordan et al., 1999). As stated above, ethanol can disrupt the cell membrane of bacteria altering its permeability allowing organic acids present in the environment to easily pass into the cell's cytoplasm, increasing cell death due to the decrease in cytoplasmic pH (Barker & Park, 2001; Jordan et al., 1999; Shapero, Nelson, & Labuza, 1978). This is what leads to the cell's inability to maintain pH homeostasis and decreasing its resistance to stress. The killing process for *E. coli* O157:H7 induced by ethanol is highly dependent on the pH of the overall environment (Barker & Park, 2001; Booth, 2003). The presence of organic acids, such as malic acid, can help protect against pathogens alongside low pH and ethanol (Semanchek & Golden, 1996). Studies done on the effect of combining low pH and ethanol shows their effectiveness in killing *E. coli* O157:H7. In this study, acid adapted *E. coli* O157:H7 was inoculated into McIlvaine buffer with adjusted pH and

ethanol additions at a target level of 7 log CFU/mL and incubated at 37°C. The addition of 5% ethanol to buffer with a pH of 3.0 showed a > 4 log CFU/mL in 5 min. At pH 4.0 and 5% ethanol, there was a 7 log CFU/mL reduction, making the *E. coli* O157:H7 undetectable after 1 hr (Jordan et al., 1999). We can conclude that pH and time play a role on the population of *E. coli* O157:H7.

To investigate the inhibitory effects of acids and ethanol on *L. monocytogenes*, cells were acid shocked in TSB-YE acidified to 3.0 or 4.0 pH with hydrochloric acid (HCl). And *L. monocytogenes* was inoculated into challenge media at pH 3.0 or 4.0 with lactate, malate, formate, sorbate, or benzoate. Formate at a concentration of 50 mM in addition with 5% ethanol was the most effective in resulting in a 5 log CFU/mL reduction in 4 mins and 10 mM of benzoate with 5% ethanol achieved a 4 log CFU/mL reduction in 5 mins (Table 4). Malate, lactate, and sorbate were less effective, but clearly, show a synergistic relationship between pH and ethanol. Based on the pKa's of the acids, it is predicted that sorbate should be more effective than formate. But it is clear that organic acids with the addition of 5% ethanol increase cell death. Using a hurdle method created multiple unfavorable conditions, the bacteria will not persist. It's important to note that the rate of cell death at a specific pH is dependent on the ethanol concentration present (Barker & Park, 2001).

Table 4. Log Reduction of *Listeria monocytogenes* at pH 3.0 and 4.0 in the presence or absence of ethanol and various organic acids (Barker & Park, 2001).

Organic Acid	Concentration (mM)	pH	EtOH (%)	Time (mins)	Log Reduction (Log CFU/mL)*
Lactate (pKa 3.86)	50	3.0	0.0	30	4.0
	50	3.0	5.0	10	4.0
	26	4.0	0.0	120	<1.0
	26	4.0	5.0	120	5.0
Malate (pKa 3.46)	50	3.0	0.0	10	4.0
	50	3.0	5.0	30	5.0
	15	4.0	0.0	120	0.0
	15	4.0	5.0	120	2.0
Formate (pKa 3.75)	50	3.0	0.0	<10	4.0
	50	3.0	5.0	5	5.0
	21	4.0	0.0	120	5.0
	21	4.0	5.0	60	5.0
Sorbate (pKa 4.76)	10	3.0	0.0	30	4.5
	10	3.0	5.0	20	5.0
	8.7	4.0	0.0	120	1.0
	8.7	4.0	5.0	120	5.0
Benzoate (pKa 4.20)	10	3.0	0.0	5	4.0
	10	3.0	5.0	10	4.0
	6.5	4.0	0.0	120	4.0
	6.5	4.0	5.0	75	5.0

*Initial load of 8.5 log CFU/mL

Deionized water was adjusted to pH 3.0 with the addition of HCl, and various ethanol concentrations were added and inoculated with *L. monocytogenes* at a concentration of 9.5 log CFU/mL. Barker and Park (2001) observed that the presence of 10% ethanol at pH 3.0 causes a greater than a 3 log CFU/mL reduction in 5 min. At 5% ethanol, there

was a greater than 5 log CFU/mL reduction within 40 min. A 2.5% ethanol concentration took more than 70 min to achieve a 4 log CFU/mL reduction. A 1.25% ethanol concentration took 90 min to achieve over a 4 log CFU/mL reduction of *L. monocytogenes*. Even in the presence of a low pH, at lower levels of ethanol there is still pathogen survival. Therefore, it is important to look at all components aiding in cider fermentation that could potentially benefit in a stronger hurdle effect.

2.7 Conclusion

Historically cider has been deemed safe due to its alcohol content and low pH. Though there have been no reported outbreaks in cider, there is also an absence of literature published on the topic of cider safety. As the cider industry grows and there is an increase in food safety regulations, research on pathogens in cider must be done to help the cider industry comply. By contrast, literature for other fermented alcoholic beverages, such as beer and wine, is more extensive than that of cider. Although similarities exist between beer, wine, and cider, many variations affect pathogen growth and survival. Many cider producers do have controls in place such as using pasteurized juice and filtering or pasteurizing final cider products, although with the possibility of pathogen presence in apples and apple juice there an increase in concern. As research has been shown, ethanol and a low pH, even in combination may not be enough to kill off pathogens present in apples, apple juice, or even cider. The lack of research does not assist in confirming the safety of cider, therefore more research is necessary to support the existing assumption exists that cider production is safe.

It is my hypothesis that the low pH and typical alcohol content of cider, this environment exerts a bactericidal and/or bacteriostatic effect on bacteria introduced

during the harvest and processing of the apples used in manufacturing the hard cider. To test this hypothesis, three common bacteria (*E. coli O157:H7*; *Salmonela sp*.; and *Listeria moncytogenes*) found in apple processing plants were assessed. *E. coli* O157:H7 has been previously linked to various apple juice outbreaks, while *Salmonella* and *L. monocytogenes* are pathogens of concern in fresh produce and can survive in acidic foods *(Diallo et al., 2011; Marler Clark 2010 ; "National Outbreak Reporting System (NORS)," 2018)*. The Specific Aims of this thesis are to:

1. Determine the survival or die off of *Escherichia coli* O157:H7, *Salmonella* spp., or *Listeria monocytogenes* during cider fermentation.

2. Determine the bactericidal/bacteriostatic effects of pH and ethanol content on *Escherichia coli* O157:H7, *Salmonella* spp., or *Listeria monocytogenes*.

3. Compare the antibacterial effects of commercial hard ciders versus a model hard cider.

3. INACTIVATION OF FOODBORNE PATHOGENS DURING CIDER FERMENTATION AND IN A CIDER MODEL SYSTEM AND COMMERCIAL CIDER

3.1 Materials and Methods

3.1.1 Bacterial Strains and Culture Preparation

Escherichia coli O157:H7, *Salmonella spp*., and *Listeria monocytogenes* bacterial strains were used for the fermentation challenge and survival studies (Table 5). Bacteria from frozen stocks stored at -70°C were streaked onto trypticase soy agar (TSA) plates and incubated at 35 ± 2°C for 24-48 h. After incubation, a single colony was placed into 10 mL trypticase soy broth supplemented with 1% glucose (TSBG) to acid adapt the cultures and incubated at 35 ± 2°C for 18 – 24 h (J. H. Ryu & Beuchat, 1998). Each bacterial strain was grown separately and repeated a second time. The three strains were combined to form cocktails of *E. coli, Salmonella,* and *L. monocytogenes.* Cells were harvested by centrifugation at 3,000 rpm for 15 min and washed three times with 0.1% peptone. Direct microscopic count (DMC) was done to determine the bacterial concentration. Each cocktail was then diluted to the target inoculum level and plated on TSA to confirm the concentration. The cocktails were then used in the fermentation or survival studies.

Table 5. Foodborne pathogen strain name, number, and source.

Strain Name	Strain Number	Isolation Information	Source[1]
E. coli O157:H7	NFPA 4211	Odwalla Apple Juice	NFL
E. coli O157:H7	NFPA 4213	Apple Cider Outbreak	NFL
E. coli O157:H7	NFPA 4219	Apple Juice Outbreak	NFL
Salmonella spp.	ATCC BAA 1045	Raw Almond Isolate	NFL
Salmonella spp.	FSL W1-030	Human Isolate	ILSIA NA
Salmonella spp.	NFPA 7201	Alfalfa Sprout Isolate	NFL
L. monocytogenes	R9-5506	Packaged Salad	ILSIA NA
L. monocytogenes	R9-5411	Caramel Apple	ILSIA NA
L. monocytogenes	R9-0506	Cantaloupe	ILSIA NA

[1]NFL – The National Food Laboratory (Livermore, CA); ILSIA NA - Institute of Life Sciences of North America (Cornell University, Ithaca, NY)

3.1.2 Fermentation Challenge

Apple juice concentrate (Fruit Smart, Grandview, WA) with an initial Brix of 70° was diluted with sterilized deionized water and the pH of the juice was adjusted with 0.1N Sodium Hydroxide (NaOH; Fisher Chemical, Pittsburgh, PA). The juice was then sterilized through a 0.22μm filter (Corning Inc., Corning, NY) and used to prepare samples containing only pathogens (OP), only yeast (OY), or pathogens and yeast (PY). Either a cocktail of *E. coli* O157:H7, *Salmonella,* or *L. monocytogenes* was used to inoculate OP and PY samples at the target inoculum level of 7 log CFU/mL. Inoculated juice was then mixed, and aliquoted into sterilized 250 mL glass bottles. Samples were then inoculated with 0.1 mL of yeast (WLP775 English Cider Yeast, White Labs, San Diego, CA). The OP samples were capped with screwcaps, and the OY and PY samples were capped with a rubber stopper and an airlock (S-type, Doc's Cellar, San Luis Obispo,

CA). The bottles were then placed in an incubator at 21 ± 1°C and sampled daily for 5 days. At time 0, before yeast addition, samples were enumerated for *E. coli* O157:H7, *Salmonella*, or *L. monocytogenes* on TSA and incubated at 35 ± 2°C for 24 h to determine the initial inoculum level. Samples were enumerated after time 0 for either *E. coli* O157:H7, *Salmonella*, or *L. monocytogenes* on TSA for samples containing no yeast or TSA + 0.1% cycloheximide (95%, ACROS Organics, Bridgewater, New Jersey) for samples containing yeast (Menz et al., 2011; Semanchek & Golden, 1996) and incubated at 35 ± 2°C for 24 – 48 h. The OY samples were measured for Brix to monitor the fermentation process. And pH was measured at time zero and Day 5 (Orion Star A211 pH meter, Pittsburg, PA), ABV was measured using an alcolyzer (Anton Paar, Graz, Austria) every day for the 5 days of fermentation.

3.1.3 *E. coli* O157:H7 Survival in A Cider Model and Commercial Cider

3.1.3.1 Cider Model

To determine the effect of pH and ABV on *E. coli* O157:H7, a cider model was created to mimic the lowest and highest pH and ABV's of cider on the market. A 0.5% (w/v) malic acid solution was made with malic acid (L(-)-Malic acid, 99%, ACROS Organics, Bridgewater, New Jersey) and 0.1% peptone. The pH of the solution was adjusted to pH 2.8, 3.0, 3.2, 3.4, 3.6, and 3.8 using 0.1N hydrochloric acid (HCL; Fisher Chemical, Pittsburgh, PA) or 0.1N NaOH. To the samples, 99.5% ethanol (Fisher Chemical, Pittsburgh, PA) was added to reach concentrations of 4, 5, 6, 7, 8, and 9% (w/v). With one pathogen, 6 pH's and 6 ABV's a total of 36 different experimental conditions were created. Control samples consisted of sterile 0.1% peptone with no pH and ethanol additions. Samples were sterilized through a 0.45μm filter (Fisherbrand,

Pittsburg, PA) and inoculated with *E. coli* O157:H7 at a target concentration of 6 log CFU/mL and stored at 21 ± 2°C.

3.1.3.2 Commercial Cider Samples

Six different ciders we purchased at a local Bevmo (Table 8), and tested for pH (Orion Star A211 pH meter, Pittsburg, PA), alcohol by volume (%) using an alcolyzer (Anton Paar, Graz, Austria), and malic acid (%) measured by titration using the AOAC method for titratable acidity (no. 942.15). Samples were then aliquoted and inoculated with *E. coli* O157:H7 at a target concentration of 6 log CFU/mL and stored at 21 ± 2°C.

3.1.3.3 Enumeration

For the model and commercial ciders samples were enumerated for *E. coli* O157:H7 on Day 0, 1, 4, and 7. During each sampling 1 mL of sample was serially diluted, pour-plated with TSA, and incubated at 35 ± 2°C for 24 h. Colonies were then counted. The limit of detection was 1 CFU/mL, therefore when bacteria were undetectable 1 CFU/mL was used to calculate log reductions.

3.1.4 Statistical Analysis

For the fermentation challenge, cider model, and commercial cider experiments, the experiments were replicated three times, with microbial counts determined in duplicate for each replication.

3.1.4.1 Cider Model and Commercial Cider

To evaluate the relationship between the mean reduction in log bacteria count and pH, ABV, and day, in the cider model, a repeated-measures analysis of variance model was estimated using JMP (Version 11, SAS Institute Inc., Cary, NC, USA). For the commercial cider, malic acid was added to the conditions analyzed and treatments were

converted into high/low categorical variables according to each variable's median value. For both the cider model and commercial cider experiments a significance level of $\alpha = 0.05$ was used to assess statistically significant relationships and differences.

3.2 Results and Discussion

This research was aimed at determining the potential for survival of foodborne bacterial pathogens during cider fermentation and post fermentation, in a cider model, and in commercial cider. While there have been no reported outbreaks directly related to pathogens in cider, however *E. coli* O157:H7 has been linked to various apple juice outbreaks, and *Salmonella* and *L. monocytogenes* are pathogens of concern in fresh produce and can survive in acidic foods (Diallo et al., 2011; Marler Clark 2010 ; "National Outbreak Reporting System (NORS)," 2018).

To assess the potential risks of these pathogens in non-fermenting and fermenting conditions, studies were conducted in which bacteria were inoculated into apple juice with an initial 8.2 ± 0.3°Brix, a pH of 3.8 prior to fermentation, and 0% ethanol present. The pathogen only – non-fermenting control samples (OP) and pathogen with yeast – fermenting experimental samples (PY) samples were inoculated with *E. coli* O157:H7 at 7.2 log CFU/mL, *Salmonella* at 7.1 log CFU/mL, and *L. monocytogenes* at 7.1 log CFU/mL. As demonstrated in **Figure 3**, during the 5-day storage period at 21±1°C of the OP samples, *L. monocytogenes* decreased by 1.4 log CFU/mL, *E. coli* O157:H7 decreased by 0.2 log CFU/mL, and *Salmonella* increased by 0.2 log CFU/mL. There was a significant difference between the initial and final population in the *E. coli* and *L. monocytogenes* control samples, but no significant difference for the *Salmonella* control

samples ($p<0.05$). These observations are expected due to the higher pH of the juice started at 3.8 and the final pH had no changed from the initial pH (**Table 6**).

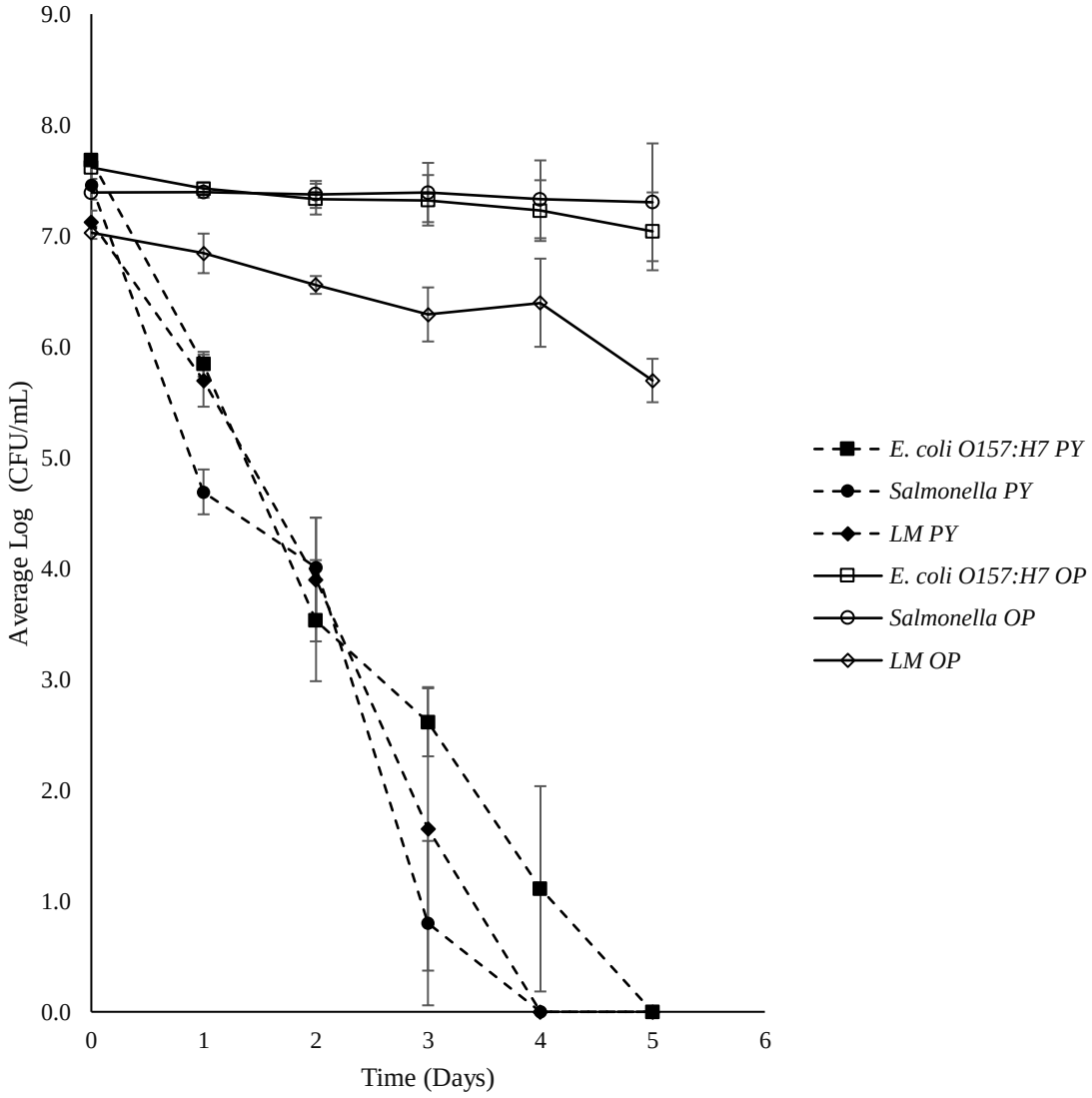

Figure 3. Ethanol Fermentation produces a potent antibacterial effect. Mean population of *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* (log CFU/mL) in cider (PY) and apple juice (OP) incubated at 21°C and sampled at Day 0, 1, 2, 3, 4, and 5. Error bars are ± standard deviations.

In a study done by Raybaudi-Massilia et al. (2009) it was observed that in apple juice with a pH of 3.94, both *E. coli* and *L. monocytogenes* populations decreased by > 1 log CFU/mL, while *Salmonella* decreased by <1 log CFU/mL. It has also been reported that *E. coli* O157:H7 survived in apple juice with a pH of 3.75 for 10 days reaching a 4 log CFU/mL reduction (Semanchek & Golden, 1996). Although it is observed in this experiment *E. coli* O157:H7 decreased in population while *Salmonella* grew in the control samples. *E. coli* O157:H7 is known for being able to acid adapt and become more resistant to stress, but *Salmonella* is also a gram-negative bacterium meaning it has characteristics similar to *E. coli. Salmonella* did not increase significantly. Raybaudi-Massilia et al. (2009) also reported that in apple juice stored at 20° C with a pH of 3.9, *Salmonella* and *L. monocytogenes* decreased by <1 log CFU/mL after 24 h, while *E. coli* O157:H7 had no change in cell population after 24 h. In addition, another study looking at apple juice with no added preservatives observed survival but not growth of E. coli O157:H7 in apple juice (pH 3.75) by more than 3 days at 25 °C (Zhao, Doyle, & Besser, 1993).

Table 6 – Fermentation had no effect on the pH of the apple juice samples. Average initial and final pH ± SD for *E. coli* O157:H7, *L. monocytogenes,* and *Salmonella* in cider and control samples (PY – Experimental samples, OP – Control samples)

	Average pH	
Sample	Initial	Final[a]
E. coli O157:H7 PY	3.8 ± 0.01	3.7 ± 0.04
L. monocytogenes PY	3.8 ± 0.01	3.7 ± 0.03
Salmonella PY	3.8 ± 0.01	3.7 ± 0.02
E. coli O157:H7 OP	3.8 ± 0.01	3.8 ± 0.05
L. monocytogenes OP	3.8 ± 0.01	3.8 ± 0.03
Salmonella OP	3.8 ± 0.01	3.8 ± 0.02

[a] Final pH was taken at Day 5

As shown in **Figure 3**, the three pathogens did not survive the 5-day fermentation period and showed a population decrease in the non-fermenting conditions. After 1-day of fermentation all PY samples showed a >1 log reduction in population. At day 2, *E. coli* had the largest log reduction of 3.7 log CFU/mL, but after 3-days of fermentation *Salmonella* decreased by 6.1 log CFU/mL and *L. monocytogenes* decreased by 5.4 log CFU/mL with an ABV of 2.0%, while *E. coli* decreased by 4.6 log CFU/mL. Semanchek & Golden (1996) found that *E. coli* O157:H7 was still detectable in cider, until 3 days of fermentation and the ABV reached 3.03%. *Salmonella* and *L. monocytogenes* were undetectable (>7 log reduction) after 4 days of fermentation with an estimated ABV of 2.3%, while *E. coli* O157:H7 survived the longest observing a 6.6 log reduction after 4 days (**Figure 3**). All the pathogens were undetectable reaching a 7.2 log reduction after 5 days with a final ABV of 4.4%. The initial pH of the cider was 3.8 while the final pH was 3.7 (**Table 6**). These experimental findings support Semanchek & Golden (2009) who reported that at the end of the 10-day storage period, *E. coli* O157:H7 stayed undetectable up to the 10-day storage period and the final ethanol concentration of the cider was 6.0% with an initial pH of 3.6 and a final pH of 3.7. Our research looked at a wider variety of pH ranges and ABV ranges to determine survival and die off of not only *E. coli* O157:H7, but also *Salmonella* and *L. monocytogenes*. There has been no research published on *Salmonella* and *L. monocytogenes* in cider, but it can be hypothesized that both pathogens may become less acid resistant and die off after the cider has reached a certain ABV, similarly to *E. coli* O157:H7.

Generally, cider is fermented from 2 – 3 weeks to a month, therefore the chance of any pathogen surviving the entirety of the fermentation process is low (A. Lea, 2016).

Primary fermentation happens during week 1 of fermentation, when most of the sugar is consumed by yeast and metabolized into ethanol and CO_2 (Meier-dörnberg, Hutzler, Michel, Methner, & Jacob, 2017). *Salmonella* and *E. coli* O157:H7 have been observed to survive in "alcohol-free" beer with ethanol adjusted to 2.5 and 5% with a pH of 4.3. This pH may be higher than that expected in cider, but in the presence of ethanol, log reduction can still be dependent on time and pH (Menz et al., 2011). And even in the presence of 5% ethanol, but at pH 7.0, *L. monocytogenes* was able to grow (1 log) in 24 h (Oh & Marshall, 1993). Although in this experiment, all three pathogens died off by the end of the 5-day fermentation period with the cider reaching an ABV of 4.4% and having a final pH of 3.7. The reduction of the pathogens is likely due to the combination of ethanol and pH or possibly due to the presence of yeast competing for nutrients. This further explains the relationship between ABV (%), pH, and time and it's effect on pathogen survival.

While it is important to determine the survival or die off *Salmonella* and *L. monocytogenes*, *E. coli* O157:H7 was chosen due to the prevalence in apple products. *E. coli* O157:H7 has also been shown to be the most acid resistant and have been shown to survive low levels of ethanol (Masuda, Hara-Kudo, & Kumagai, 2016; Menz et al., 2011). Therefore, *E. coli* can be used to conservatively predict how other bacteria might behave in the same environment. To better understand the effects of pH and ABV in combination on *E. coli* O157:H7, a cider model was created with a base of 0.5% malic acid adjusted to six pH's in combination with six ethanol concentrations. *E. coli* O157:H7 was acid adapted by being grown in TSBG, in order to create a more conservative measurement, in the case that apple juice or cider is contaminated with naturally acid-

adapted cells. In samples at ABV 4 and 5% EtOH at pH 3.6 and 3.8 there was a <2 log reduction observed at day 1 (**Table 7a**). The control had an initial 1.7 log reduction on Day 0, but all the samples with adjust pH and ABV showed values less than 1.0 log reduction ranging from 0.0-0.9 log CFU/mL. After 1-day, at ABV 7, 8, and 9% from pH 2.8 – 3.6 there was a ≥6 log reduction in *E. coli* population observed (**Table 7a**). Jordan et al. (1999) observed a 7-log reduction of *E. coli* O157:H7 within 1 h of incubation in McIlvaine buffer at pH 4.0 and 5% ethanol. The acid used to adjust the pH in that study was lactate, and the pKa of lactate is 3.85 being close to 4.0 the amount of dissociated acids being present and being able to damage cell membranes, DNA, and proteins. As shown in **Figure 4a** and **Figure 4b**, at day 4, there was a log reduction of >5 log reduction observed at all ABV levels and the lower pH range of 2.8 – 3.2. Once the pH started to increase, with the lower ABV levels there was more survival that can be seen in the reduction patterns outlined in **Table 7b**. A log reduction of >6 was observed at 8 and 9% ABV for pH 3.6 and 3.8 but all levels below still showed survival at day 4. At day 7, all pH and ABV combinations had log reductions of >6, except for 4% ABV at pH 3.6 and 3.8 which resulted in a 5.8 and 5.7 log reduction, respectively (**Table 7c**). It was observed that the lower the pH and the higher the ABV the quicker the die off. Menz et al. (2011) acid adapted *E. coli* O157:H7 and observed in a solution with 2.7% ethanol and a pH of 4.3 there was a decrease in population by 3 log CFU/mL in 40 days; at 5% ethanol there was a decrease in population by 3 log CFU/mL in 30 days. The relationship between pH and ABV, pH and day, ABV and day, and day, pH and ABV are all statistically significant ($p<0.05$). Meaning the effect of each treatment on the log reduction is dependent on one another, for example, the effect that pH was estimated to

have on the log reduction depended on the day and the ABV at that time, indicating the presence of ethanol alone and keeping malic acid constant, isn't enough to kill the bacterial population (Appendix C, E, F). This means that factors such as time and pH also need to be taken into consideration.

Table 7a. Effect of ABV and pH on Log Reductions (CFU/mL) at Day 1 on *E. coli* O157:H7. Average *E. coli* O157:H7 log reductions (CFU/mL) sampled from all combinations of pH and ABV (%) at **Day 1** incubated at 21 ± 1°C.

ABV (%)	pH					
	2.8	**3.0**	**3.2**	**3.4**	**3.6**	**3.8**
4	3.6	3.9	3.5	2.8	1.7	1.0
5	5.2	4.7	4.2	4.0	1.7	1.8
6	6.0	5.6	4.5	5.0	3.5	3.6
7	6.6	6.6	6.6	6.6	6.0	4.1
8	6.6	6.6	6.6	6.6	6.1	5.0
9	6.6	6.6	6.6	6.6	6.6	6.1

≥ 6.0 log < 6 - 3.0 log < 3.0 log

Table 7b. Effect of ABV and pH on Log Reductions (CFU/mL) at Day 4 on *E. coli* O157:H7. Average *E. coli* O157:H7 log reductions (CFU/mL) sampled from all combinations of pH and ABV (%) at **Day 4** incubated at 21 ± 1°C.

ABV (%)	pH					
	2.8	**3.0**	**3.2**	**3.4**	**3.6**	**3.8**
4	6.1	5.7	5.0	4.9	4.1	4.5
5	6.3	6.3	6.0	5.3	4.2	4.3
6	6.3	6.3	6.3	6.3	5.9	4.9
7	6.6	6.6	6.6	6.6	6.1	5.4
8	6.6	6.6	6.6	6.6	6.6	6.1
9	6.6	6.6	6.6	6.6	6.6	6.6

≥ 6.0 log < 6 - 3.0 log < 3.0 log

Table 7c. Effect of ABV and pH on Log Reductions (CFU/mL) at Day 7 on *E. coli* O157:H7. Average *E. coli* O157:H7 log reductions (CFU/mL) sampled from all combinations of pH and ABV (%) at **Day 7** incubated at 21 ± 1°C.

ABV (%)	pH					
	2.8	**3.0**	**3.2**	**3.4**	**3.6**	**3.8**
4	6.3	6.3	6.3	6.3	5.8	5.7
5	6.3	6.3	6.3	6.3	6.3	6.0
6	6.3	6.3	6.3	6.3	6.3	6.3
7	6.6	6.6	6.6	6.6	6.6	6.6
8	6.6	6.6	6.6	6.6	6.6	6.6
9	6.6	6.6	6.6	6.6	6.6	6.6

≥ 6 log < 6 - 3.0 log < 3 log

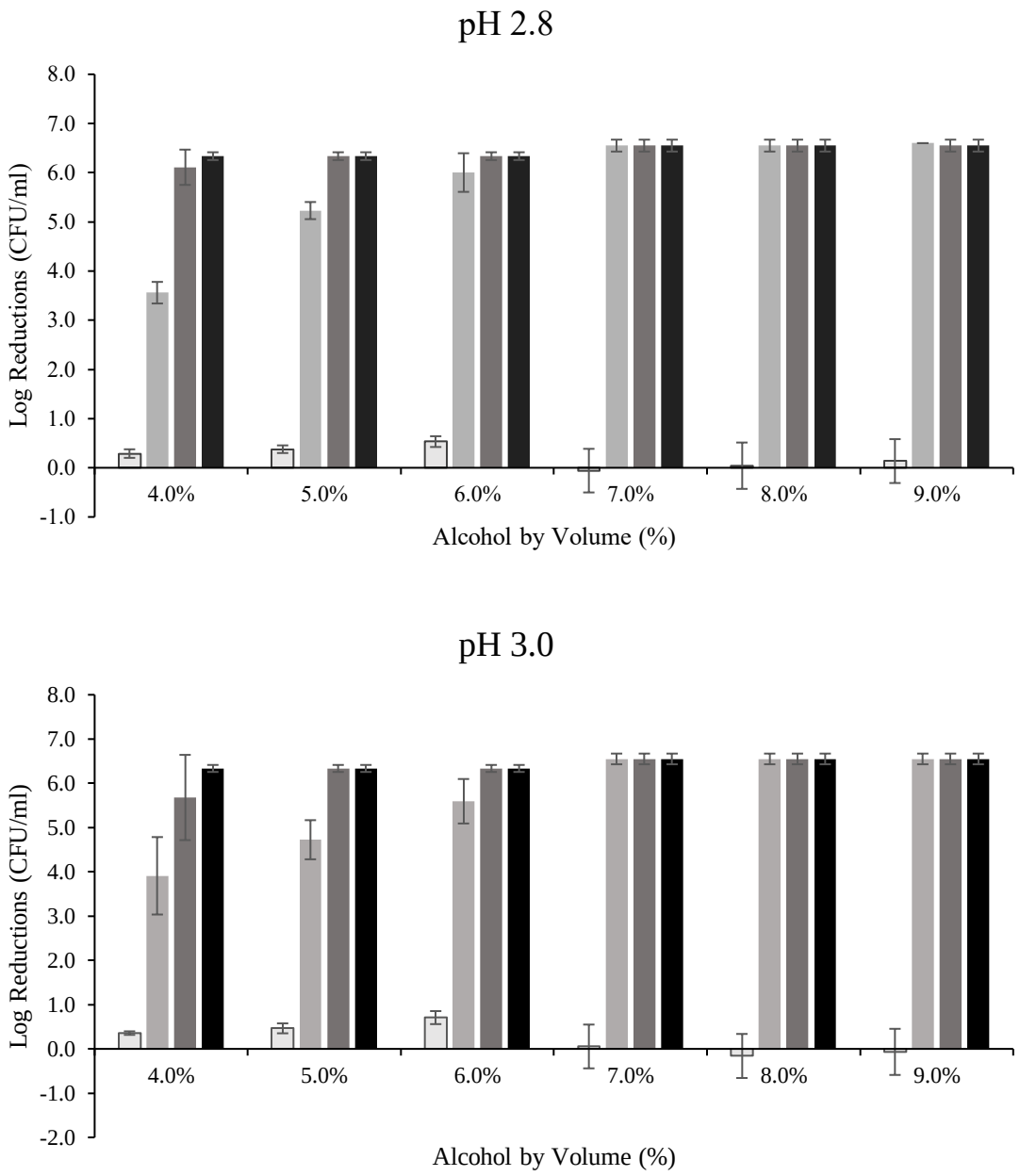

Figure 4a. Average log reductions (CFU/mL) of *E. coli* O157:H7 at pH 2.8 and 3.0 and various ABV (4.0 – 9.0%) stored at 21°C, at Day 0, 1, 4, and 7 (□ D0 ■ D1 ■ D4 ■ D7). Error bars are ± standard deviations.

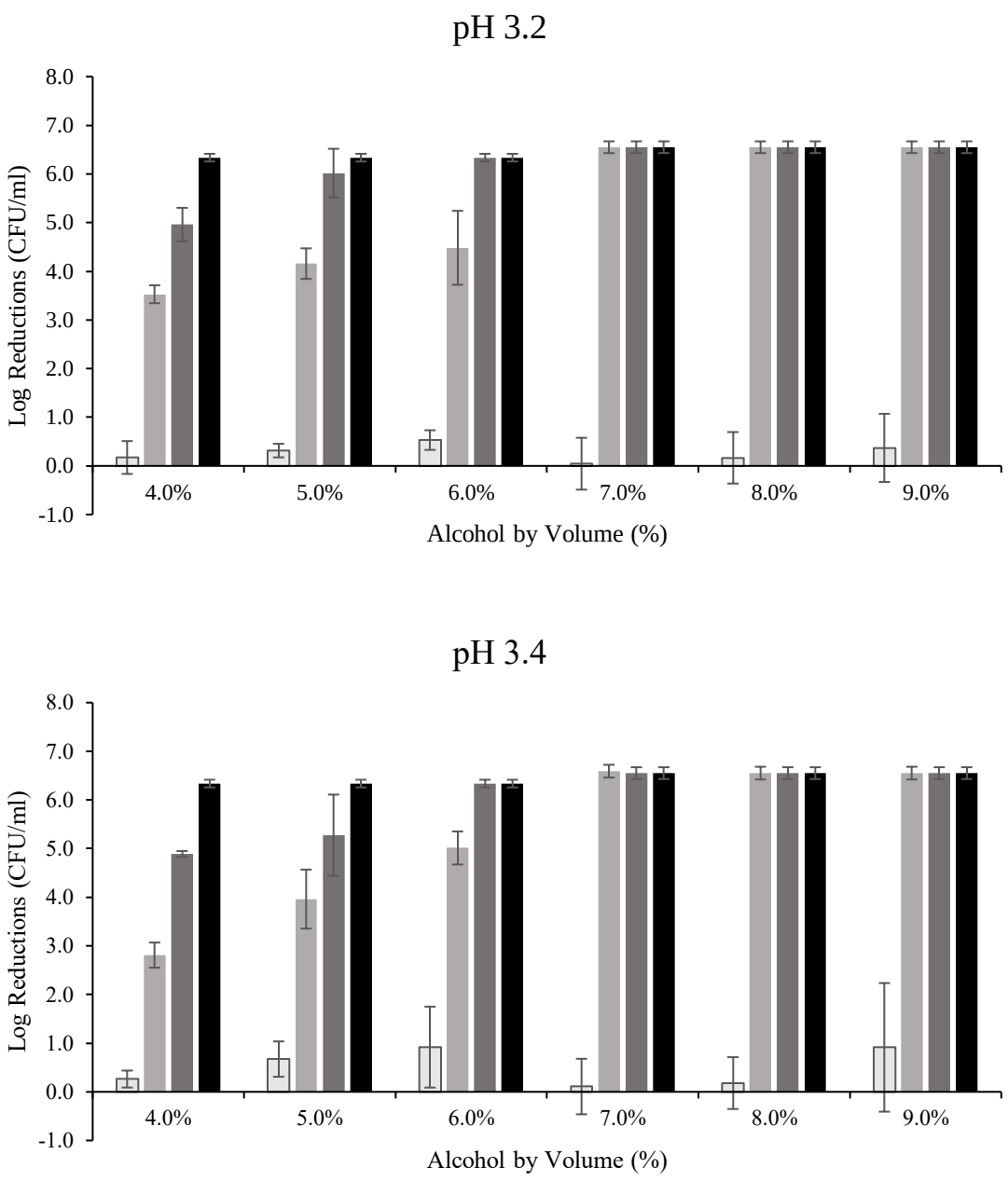

Figure 4b. Average log reductions (CFU/mL) of *E. coli* O157:H7 at pH 3.2 and 3.4 and various ABV (4.0 – 9.0%) stored at 21°C, at Day 0, 1, 4, and 7 (□ D0 ■ D1 ■ D4 ■ D7). Error bars are ± standard deviations.

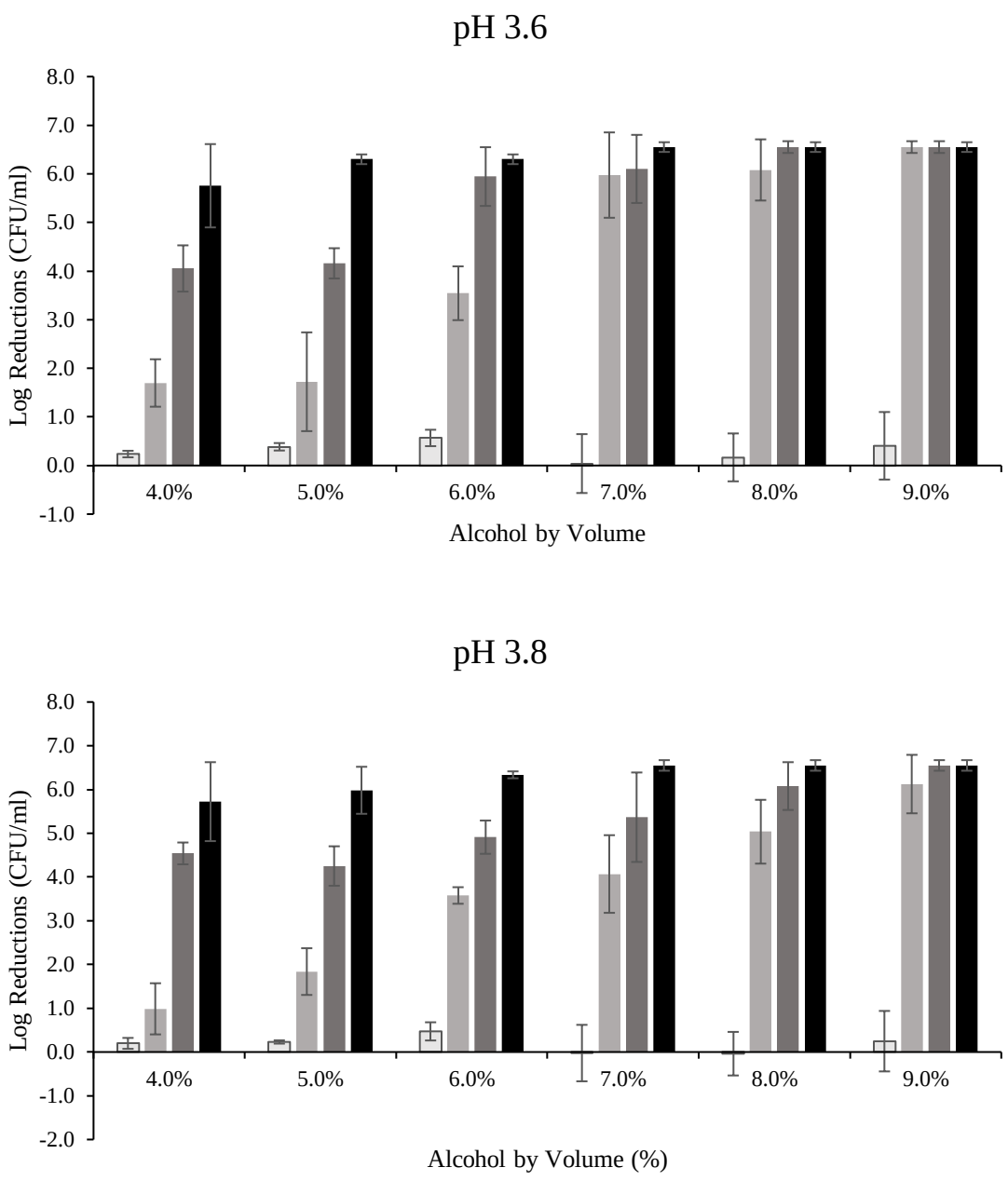

Figure 4c. Average log reductions (CFU/mL) of *E. coli* O157:H7 at pH 3.6 and 3.8 and various ABV (4.0 – 9.0%) stored at 21°C, at Day 0, 1, 4, and 7 (□ D0 ■ D1 ■ D4 ■ D7). Error bars are ± standard deviations.

The pH for our cider model was adjusted with HCl. Because HCl is a strong acid, it is mostly dissociated and therefore for this model system experiment the log reductions may be more conservative because HCl is less effective, at the same pH, in preventing bacterial growth. Malic acid is a weak acid and does not completely dissociate in water. The undissociated form left behind is able to pass through the cell membranes preventing bacterial growth (Wang et al., 2018). Although the mechanism of dissociated versus undissociated mobility through the cell membrane is not fully understood. Cider producers generally achieve their desired pH based on the apple variety juice used, or the addition of malic acid. For the initial juice, the titratable acid in g/L malic acid should be around 0.3 – 0.7%, and the ideal pH should be around 3.2 – 3.8 (A. Lea, 2016). The antimicrobial effects of acids on foodborne pathogens have been previously observed (Barker & Park, 2001; Buchanan & Edelson, 1996; Miller & Kaspar, 1994; Raybaudi-Massilia et al., 2009; J. H. Ryu & Beuchat, 1998). Raybaudi-Massilia et al. (2009) looked at the effect of malic acid in various fruit juices, including apple with a pH of 3.9. After 24 h of storage with 0.4, 0.5, and 0.6% malic acid, *L. monocytogenes* was no longer detectable reaching an 8 log CFU/mL reduction at all three concentrations of malic acid. For *Salmonella* Enteritidis, there was a <3 log CFU/mL reduction, 5 log CFU/mL reduction, and not detectable (8 log CFU/mL reduction) at 0.4, 0.6, and 0.8% malic acid, respectively. *E. coli* O157:H7 showed a similar pattern with a <2 log CFU/mL reduction, <5 log CFU/mL reduction, and not detectable (8 log CFU/mL reduction) after 24 hrs of incubation at 0.4, 0.6, and 0.8% malic acid, respectively. The pH of the juice at 0.4% malic acid was 3.3, 0.6% was 3.1, and 0.8% was 3.1. As stated prior, some acids are more effective than others, depending on the ratio of the dissociated to undissociated

compounds present in solution, and pH level. The survival of *E. coli* O157:H7 and *Salmonella* Enteritidis at 0.4 and 0.6% malic acid, indicate that alone, malic acid may not be enough to kill off the pathogens (Raybaudi-Massilia et al., 2009). In order to further identify the antimicrobial effects of cider, commercial cider was inoculated with *E. coli* O157:H7.

Comparing observations in the cider model, similar inactivation patterns were true for *E. coli* O157:H7 in commercial cider. Six different commercial ciders with various alcohol contents (4.3 – 9.6% ABV) were inoculated with *E. coli* O157:H7. As presented in **Table 8**, commercial ciders were measured for ABV, pH, and total acid (g/L malic acid) prior to inoculation.

Table 8. Analysis of Commercial Ciders demonstrates substantial variability in product characteristics. Shown are the ABV, pH, and malic acid (%, g/L) of six commercial cider samples. ABV ranged from 4.3 to 9.6%; pH ranged from 3.2 to 3.7; while malic acid ranged from 0.58 to 0.90%.

Sample	Cider	ABV (%)	pH	Malic Acid (%)
EA	Easy Apple Angry Orchard	4.3	3.4	0.58
CA	Crisp Apple Angry Orchard	5.0	3.2	0.68
BD	Brooks Dry Cider	6.2	3.7	0.65
RR	Rambling Route Hard Cider	6.7	3.7	0.73
SC	See Canyon Classic	8.7	3.7	0.90
BONE	See Canyon Boneyard	9.6	3.7	0.85

In the control samples which contained peptone, the *E. coli* population grew by 1.8 log CFU/mL in the 7-day period. *E. coli* O157:H7 died off in all cider samples by the end of the 7-day period and pH, ABV and day were all significantly associated with the decrease in bacteria ($p<0.05$). After 1 day in CA, SC, and BONE samples *E. coli* was undetectable reaching a >6 log reduction. The CA sample had an ABV of 5%, although low, in combination with a pH of 3.2 resulted in a quick population die off. And it is

expected that SC and BONE samples with the highest ABV levels would result in the quickest die off due to having the highest ABV (%) (**Table 9**). In the other samples, EA, BD, and RR, there was a 3.8, 3.7, and 4.9 log reduction after 1 day, respectively. EA has an ABV of 4.3% and a pH of 3.4, BD has an ABV of 6.2% and a 6.7 pH, and RR has an ABV of 6.7% and a pH of 3.7. It is observed that at some point in time pH plays a bigger role when the ABV is smaller. An interaction between ABV and day was found to be statistically significant, meaning that the effect that day had on the outcome differed depending on the ABV level. In all samples, *E. coli* became undetectable reaching a >6 log reduction after 4 days and stayed undetectable through day 7 (**Figure 5**). It has been previously observed that the killing process by ethanol for *E. coli* O157:H7 is pH dependent (Jordan et al., 1999). CA had the lowest pH, possibly explaining the rapid *E. coli* O157:H7 die off despite the lower ABV. Although, malic concentration (%) was not significantly associated with the outcome variable, after also controlling for pH, ABV, and day ($p>0.05$). But this doesn't mean it did not have an effect on log reductions, but rather after looking at the effect of pH and ABV (%) on log reduction, malic acid (%) doesn't further help explain log reduction in relation to time. Type of acid may be relevant but the rate of cell death at a specific pH is dependent on ethanol concentration and time (Barker & Park, 2001). Studies have shown that 0.8% malic acid in apple juice with a pH of 3.1 is effective at killing *E. coli* O157:H7, *Salmonella* Enteritidis, and *L. monocytogenes* after 1 day (Raybaudi-Massilia et al., 2009).

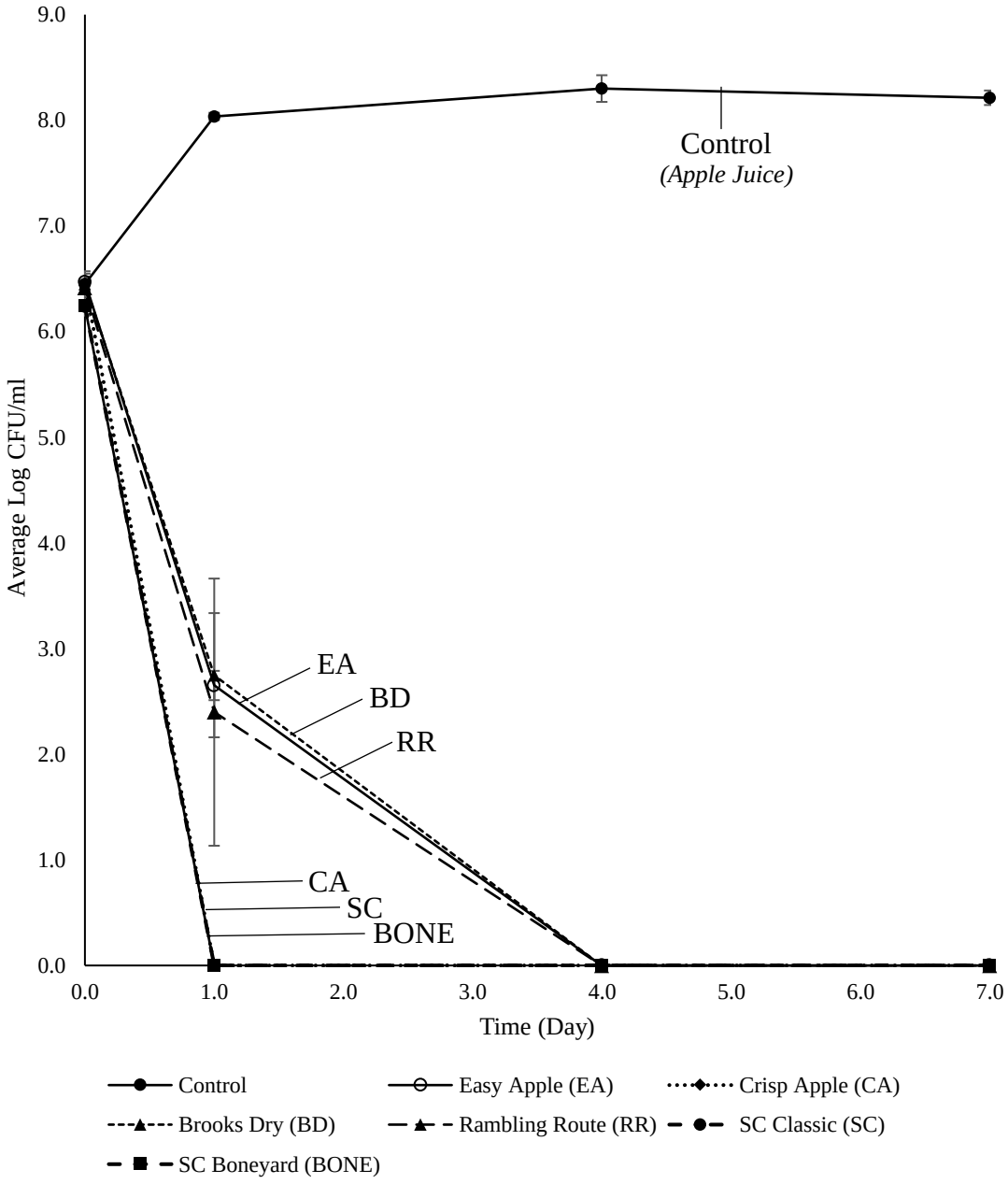

Figure 5. Commercial ciders demonstrate variable anti-bacterial (*E. coli* O157:H7) efficacy. Average log CFU/mL of *E. coli* O157:H7 in commercial cider incubated at 21°C for 7 days. Crisp Apple, SC Classic, and SC Boneyard reached a >6 log reduction by 1 day and was still undetectable through Day 7. Error bars are ± standard deviations.

There is little information published on the effects of pH and ethanol in combination on foodborne pathogens, although observations show that both work synergistically to inhibit pathogen survival or growth (Jordan et al., 1999). In an environment with a low pH, the addition of ethanol will aid in sensitizing cells to osmotic stress by altering membrane permeability. This membrane disruption interferes with distribution of solutes in the cytoplasm by increasing the passage of protons, organic acids, and other osmotic solutes (Barker & Park, 2001).

The sensitivity of pathogens, and more specifically *E. coli* O157:H7, is increased by a low pH, the presence of ethanol, and time of exposure. Menz et al. (1996) reported that *E. coli* O157:H7 was undetectable in "alcohol free beer" with a pH of 4.3, and an adjusted ABV of 5% in 30 days. The pH of 4.3 is still fairly high in comparison to cider; as stated above the desirable pH range for cider is 3.2 – 3.8 (A. Lea, 2016). Looking at a similar ethanol concentration ranges for the commercial ciders, it is observed that pH has an effect on the log reductions. CA (5.0% ABV) which is in the lower end of the samples, observed a >6.0 log reduction at 1 day of storage. In comparison to BD (6% ABV) and RR (7% ABV) reached log reductions of 3.7 and 4.0 log CFU/mL, respectively. It is expected that at higher ABV's there will be larger log reductions observed; at 1-day SC and BONE had a >6.0 log reductions, with both samples having a pH of 3.7. Malic acid concentration (g/L) was measured in each of the cider samples and ranged from 0.58 – 0.90%.

The statistical relationship between pH, ABV, and time shown in the cider model observations indicate that individually, and together each factor plays a role in the inactivation of *E. coli* O157:H7. Masuda et al. (2016) showed that in an environment

containing 2.5% ABV, there was no decrease in *E. coli* O157:H7 presence in 9 days at both 18 and 30°C. Although at 5% ABV, the population decreased by 2.0 log CFU/mL in 9 days, but the pH of the sample was 7.0 (Masuda et al., 2016). It is important to observe *E. coli* O157:H7 in acidic environments because acid tolerance is an important component of virulence for this bacterium (Leyer, Wang, & Johnson, 1995). The infective dose for *E. coli* O157:H7 is as few as 10 – 100 cells, therefore the presence of any cells could potentially transmit pathogens through the cider. The optimal growth pH range for *E. coli* O157:H7 is between 5.0 – 7.0 but can survive pH's lower than that. The mechanism in which bacteria can become acid tolerant is related to the ability of the cells to repair damage to DNA caused by H+ (J. H. Ryu & Beuchat, 1998). Therefore, the effect of ethanol on *E. coli* O157:H7 even at neutral pH is still effective in decreasing the bacterial population in relation to time. This can help explain the ≥6.0 log reduction at pH 2.8 and at 5% ABV after 1 day, while other samples at the same ABV (%) did not reach a 6.0 log reduction in the same amount of time. Looking at the effect of ethanol, pH and malic acid content, they work synergistically to kill off any potential bacterial pathogen population present in cider. As stated above, malic acid alone is not enough to kill off bacterial pathogens, although the addition of more malic acid to cider would be beneficial. Looking at the malic acid content of the SC cider (0.90%) is fairly high but would aid in killing bacterial pathogens. The purpose of adding malic acid is general for final flavor modification. Therefore, addition of malic acid, if not negatively changing the flavor of the desired cider, will decrease pH and work in combination with pH and ethanol to kill pathogens.

In this work, the survival and die off of *E. coli* O157:H7, *Salmonella,* and *L. monocytogenes* was investigated during the cider fermentation process. And the fate of *E. coli* O157:H7 was evaluated in a cider model and commercial cider. *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* did not survive the 5-day fermentation period. In the cider model, as expected at higher levels of ethanol and lower pH's will result in more rapid die off of *E. coli* O157:H7, although there was no pathogen survival after the 7-day storage period for all combinations of pH and ABV. For the commercial cider, *E. coli* O157:H7 was undetectable in all samples after 4 days. Therefore, during cider production, pH should be monitored, as well as final ABV and storage time in order to ensure a safe final product.

4. FUTURE RESEARCH

This research only assesses the lowest ABV of 4% and does not speak to anything lower than that. Therefore, cider makes producing cider with a less than 4% ABV would need to run addition microbial tests. But future research assessing the fate of foodborne pathogens in cider could be done by manipulating only malic acid concentrations. Further research into looking at the bactericidal effects of malic acid in cider during and post fermentation could be beneficial for cider producers granted changing the malic acid concentration of juice or cider would not affect the final product.

These same experiments should be evaluated at various temperatures as well. Storage environments for finished products are not always controlled and could be stored at refrigerated temperatures which may be more favorable for pathogen survival. The cider model and commercial cider experiments can also be done with other foodborne pathogens, such as *Salmonella* spp. and *Listeria monocytogenes* to further ensure safety.

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APPENDICES

APPENDIX A. TAX CLASSIFICATIONS AS DEFINED BY THE ALCOHOL AND TOBACCO TAX AND TRADE BUREAU

Table A1. The tax classifications for cider and perry as defined by the Alcohol and Tobacco Tax and Trade Bureau (TTB, 2018)

Tax Classifications for Cider/Perry Products	Per wine gallon
Still Wine Tax Classes (0.396 g CO2/100 mL of less)	
≤ 14% ABV	$1.07
> 14 - 21% ABV	$1.57
> 21 - 24% ABV	$3.15
Artificially Carbonated Wine Tax Class	$3.30
Sparkling Wine Tax Class	$3.40
Hard Cider Tax Class*	22.6¢

*No more than 0.64g CO2/100mL; derived primarily from apples/pears or apple/pear juice concentrate and water; containing no other fruit product or fruit flavoring other than apple/pear, and containing at least 0.5% and less than (not equal to) 8.5% alcohol by volume

APPENDIX B. RAW DATA FOR *E. COLI* O157:H7, *SALMONELLA,* AND *L. MONOCYTOGENES* IN FERMENTING CIDER AND APPLE JUICE

Table B1. Mean log reduction for *E. coli* O157:H7, *Salmonella,* and *L. monocytogenes* (CFU/mL) in fermenting cider and apple juice (control) for Day 0, 1, 2, 3, 4, and 5, stored at 21°C.

	Average Log Reduction CFU/mL					
Sample	T0	T1	T2	T3	T4	T5
E. coli O157:H7 Cider	-0.4	1.4	3.7	4.6	6.6	7.2
L. monocytogenes Cider	0.0	1.4	3.2	5.4	7.1	7.1
Salmonella Cider	-0.4	2.4	3.1	6.1	7.1	7.1
E. coli O157:H7 Control[a]	-0.4	-0.2	-0.1	-0.1	0.0	0.2
L. monocytogenes Control[a]	0.1	0.3	0.6	0.8	0.7	1.4
Salmonella Control[a]	-0.3	-0.3	-0.3	-0.4	-0.3	-0.2

[a] Apple juice, no yeast added

APPENDIX C. RAW DATA FOR *E. COLI* O157:H7 IN A CIDER MODEL

Table C1. Average log reduction of *E. coli* O157:H7 (CFU/mL) for the cider model at Day 0, 1, 4, and 7, stored at 21°C.

		Average Log Reduction (CFU/mL)			
pH	ABV (%)	T0	T1	T4	T7
2.9[a]	0.0%	1.7	-1.5	-1.8	-1.8
2.8	4.0%	0.3	3.6	6.1	6.3
2.8	5.0%	0.4	5.2	6.3	6.3
2.8	6.0%	0.5	6.0	6.3	6.3
2.8	7.0%	-0.1	6.6	6.6	6.6
2.8	8.0%	0.0	6.6	6.6	6.6
2.8	9.0%	0.1	6.6	6.6	6.6
pH	ABV (%)	T0	T1	T4	T7
3.0	4.0%	0.4	3.9	5.7	6.3
3.0	5.0%	0.5	4.7	6.3	6.3
3.0	6.0%	0.7	5.6	6.3	6.3
3.0	7.0%	0.1	6.6	6.6	6.6
3.0	8.0%	-0.2	6.6	6.6	6.6
3.0	9.0%	-0.1	6.6	6.6	6.6
pH	ABV (%)	T0	T1	T4	T7
3.2	4.0%	0.2	3.5	5.0	6.3
3.2	5.0%	0.3	4.2	6.0	6.3
3.2	6.0%	0.5	4.5	6.3	6.3
3.2	7.0%	0.0	6.6	6.6	6.6
3.2	8.0%	0.2	6.6	6.6	6.6
3.2	9.0%	0.4	6.6	6.6	6.6
pH	ABV (%)	T0	T1	T4	T7

3.4	4.0%	0.3	2.8	4.9	6.3
3.4	5.0%	0.7	4.0	5.3	6.3
3.4	6.0%	0.9	5.0	6.3	6.3
3.4	7.0%	0.1	6.6	6.6	6.6
3.4	8.0%	0.2	6.6	6.6	6.6
3.4	9.0%	0.9	6.6	6.6	6.6
pH	ABV (%)	T0	T1	T4	T7
3.6	4.0%	0.2	1.7	4.1	5.8
3.6	5.0%	0.4	1.7	4.2	6.3
3.6	6.0%	0.6	3.5	5.9	6.3
3.6	7.0%	0.0	6.0	6.1	6.6
3.6	8.0%	0.2	6.1	6.6	6.6
3.6	9.0%	0.4	6.6	6.6	6.6
pH	ABV (%)	T0	T1	T4	T7
3.8	4.0%	0.2	1.0	4.5	5.7
3.8	5.0%	0.2	1.8	4.3	6.0
3.8	6.0%	0.5	3.6	4.9	6.3
3.8	7.0%	0.0	4.1	5.4	6.6
3.8	8.0%	0.0	5.0	6.1	6.6
3.8	9.0%	0.2	6.1	6.6	6.6

[a] Control samples made with 0.1% peptone, pH was not adjusted, no ethanol additions

APPENDIX D. RAW DATA FOR *E. COLI* O157:H7 IN COMMERCIAL CIDER

Table D1. Raw averaged *E. coli* O157:H7 log reduction (CFU/mL) in commercial cider with various pH and ABV (%), at Day 0, 1, 4, and 7, stored at 21°C.

	Log Reduction (CFU/mL)			
Cider	T0	T1	T4	T7
Control	0.0	-1.6	-1.9	-1.8
Easy Apple (EA)	-0.1	3.8	6.4	6.4
Crisp Apple (CA)	0.0	6.4	6.4	6.4
Brooks Dry (BD)	0.0	3.7	6.4	6.4
Rambling Route (RR)	0.0	4.0	6.4	6.4
SC Classic (SC)	0.2	6.4	6.4	6.4
SC Boneyard (BONE)	0.2	6.4	6.4	6.4

APPENDIX E. LEAST MEAN SQUARES OF *E. COLI* O157:H7 POPULATION REDUCTIONS GENERATED IN JMP WITH REPEATED MEASURES ANALYSIS

Mean log reduction of *E. coli* O157:H7 (CFU/mL) based on least mean squares generated from repeated measures analysis of variance ($p<0.05$) at Day 1

% ETOH	Average Log Reduction (CFU/mL)[a]					
	pH					
	2.8	**3.0**	**3.2**	**3.4**	**3.6**	**3.8**
4	3.6JI	3.3KJ	3.5HGFI	2.8KL	2.2ML	1.4N
5	5.2DCE	4.7DGFE	4.2HGFI	4.3HGF	2.1M	1.8MN
6	6.0AB	5.4BC	4.6GFE	4.7DFE	3.2KJ	3.6HJI
7	6.6A	6.6A	6.6A	6.0AB	6.0A	4.1HGI
8	6.6A	6.6A	6.6A	6.6A	6.2A	5.3DC
9	6.6A	6.6A	6.6A	6.6A	6.6A	6.2A

Mean log reduction of *E. coli* O157:H7 (CFU/mL) based on least mean squares generated from repeated measures analysis of variance ($p<0.05$) at Day 1

% ETOH	Average Log Reduction (CFU/mL)[a]					
	pH					
	2.8	**3.0**	**3.2**	**3.4**	**3.6**	**3.8**
4	6.2ABC	6.0BCD	5.0F	4.9F	3.9G	3.9G
5	6.3AB	6.3AB	6.0BCD	5.8DC	5.2EF	3.6G
6	6.3AB	6.3AB	6.3AB	6.3AB	5.9BCD	4.8F
7	6.6A	6.6A	6.6A	6.6A	6.6A	5.5DE
8	6.6A	6.6A	6.6A	6.6A	6.6A	6.6A
9	6.6A	6.6A	6.6A	6.6	6.6A	6.6A

Mean log reduction of *E. coli* O157:H7 (CFU/mL) based on least mean squares generated from repeated measures analysis of variance ($p<0.05$) at Day 1

% ETOH	Average Log Reduction (CFU/mL)[a]					
	pH					
	2.8	**3.0**	**3.2**	**3.4**	**3.6**	**3.8**
4	6.3A	6.3A	6.3A	6.3A	6.2A	5.7B
5	6.3A	6.3A	6.3A	6.3A	6.3A	6.0B
6	6.3A	6.3A	6.3A	6.3A	6.3A	6.3A
7	6.6A	6.6A	6.6A	6.6A	6.6A	6.1A
8	6.6A	6.6A	6.6A	6.6A	6.6A	6.6A
9	6.6A	6.6A	6.6A	6.6A	6.6A	6.6A

[a] Values that are followed by the same letter are not significantly different ($p > 0.05$)

APPENDIX F. STATISTICAL ANALYSIS FOR CIDER MODEL

Preliminary Results of Cider Data Analysis for Katy Yamada

A repeated measures analysis of variance, estimating and testing for an association between the reduction in log bacteria count and pH, ABV, and day was estimated. pH, ABV and day were all significantly associated with the decrease in bacteria.

All interactions were statistically significant. This means that, for example, the effect that pH was estimated to have on the log reduction depended on what day it was and what the ABV value for that sample was. The graphs above try to illustrate the complex relationships that were discovered.

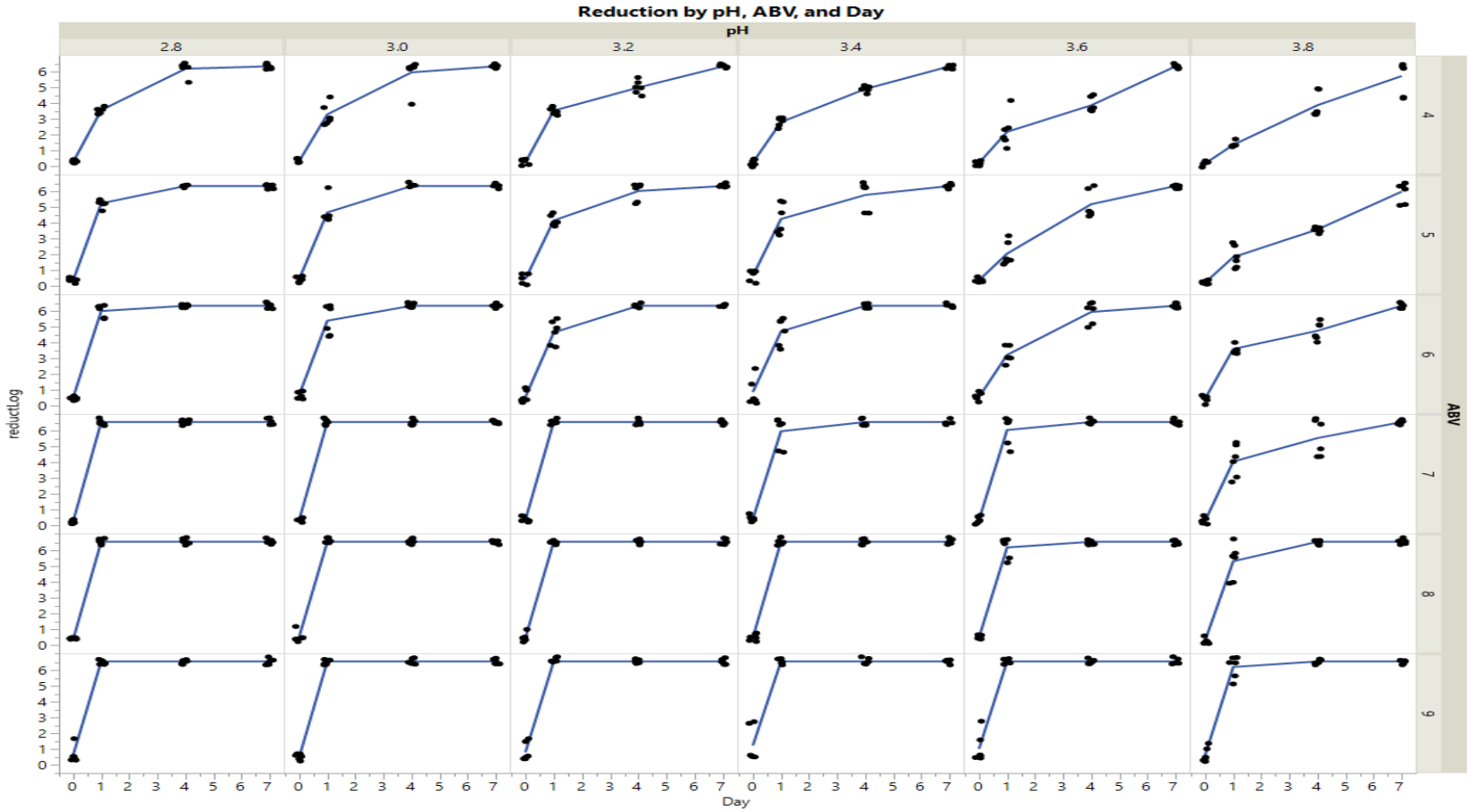

Figure F1. Visual graph to represent the relationship between pH, ABV, and Day ran through JMP with repeated measures analysis of variance of cider model data

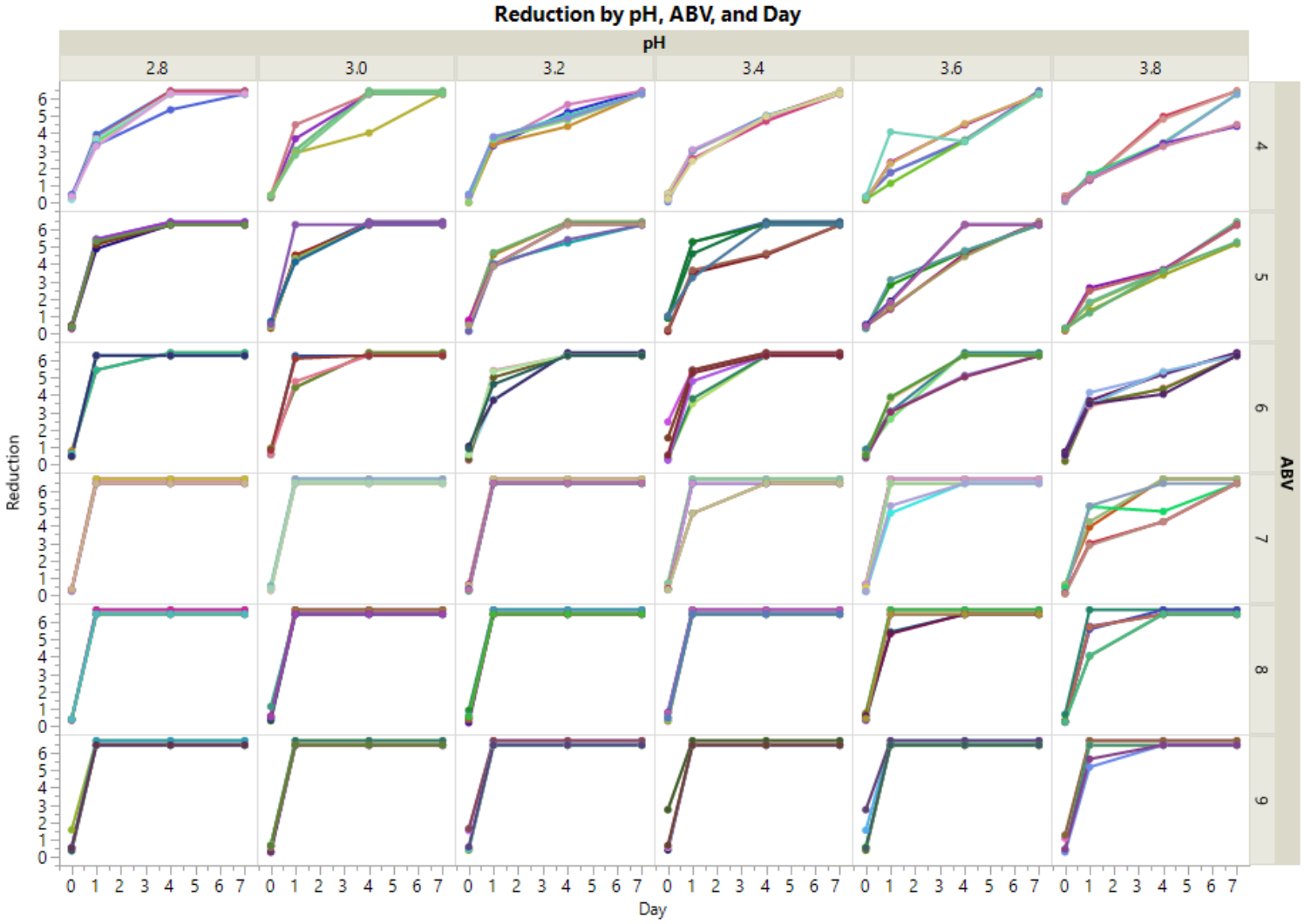

Figure F2. Extra visual image of data points graphed by pH, ABV, and Day for cider model data

Table F1. Fixed effects tests ran in JMP with repeated measures analysis of variance with cider model data

Fixed Effects Test			
Source	DFNum	F Ratio	Prob > F
Day	3	19613.2	<0.0001
pH	5	56.4	<0.0001
ABV	5	183.8	<0.0001
pH*ABV	25	5.3	<0.0001
Day*pH	15	29.0	<0.0001
Day*ABV	15	83.3	<0.0001
Day*pH*ABV	75	4.7	<0.0001

APPENDIX G. STATISTICAL ANALYSIS FOR COMMERCIAL CIDER

Commercial Cider Data:

The pH, ABV, and % malic were converted into high/low categorical variables according to each variable's median value. This was done because these variables values were not actual treatment conditions, as in the experimental data case, and thus the data was not balanced in terms of these variables. It is also easier to visualize the data in this fashion. Finally, if we treat these variables as categorical, as high/low, we don't have to assume there is a linear association between their values and the outcome variable. Since two tubes were taken from each bottle, and these measurements would be strongly correlated with one another, these tubes' measurements were averaged to simplify the statistical analysis needed for the data. Therefore, the outcome variable analyzed is the average bacteria reduction for the two tubes of each bottle. Day 7 measurements were eliminated from the analysis as Day 4 and Day 7 measurements were identical for all tubes.

A repeated measures analysis of variance, estimating and testing for an association between the mean reduction in bacteria and pH, ABV, malic, and day was estimated. pH, ABV and day were all significantly associated with the decrease in bacteria. % malic was not significantly associated with the outcome variable, after also controlling for pH, ABV, and day.

An interaction between ABV category and day was found to be statistically significant. That means that the effect that day had on the outcome differed depending on the ABV level. Similarly, one could say that the effect that ABV had on the outcome differed depending on the day.

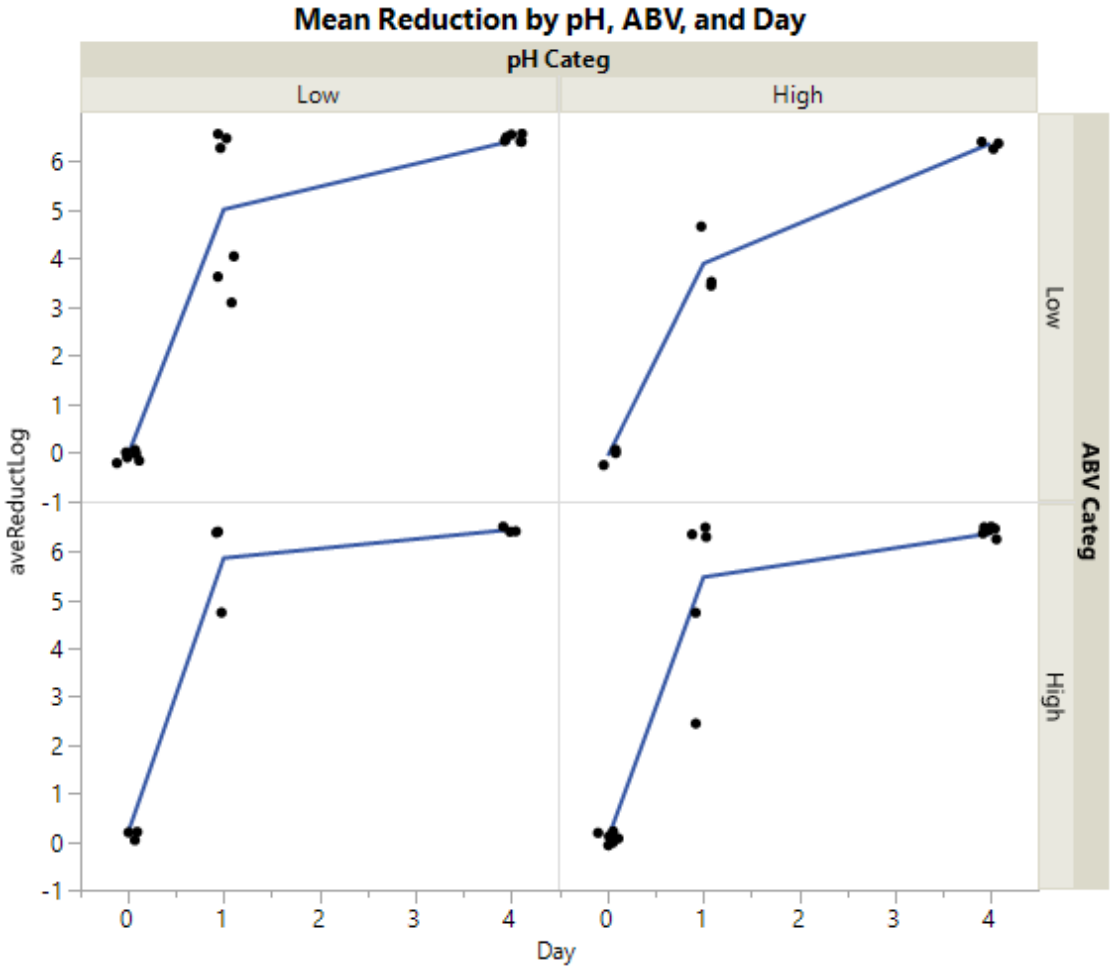

Figure G1. Visual graph to represent the relationship between pH, ABV, and Day ran through JMP with repeated measures analysis of variance of commercial cider data

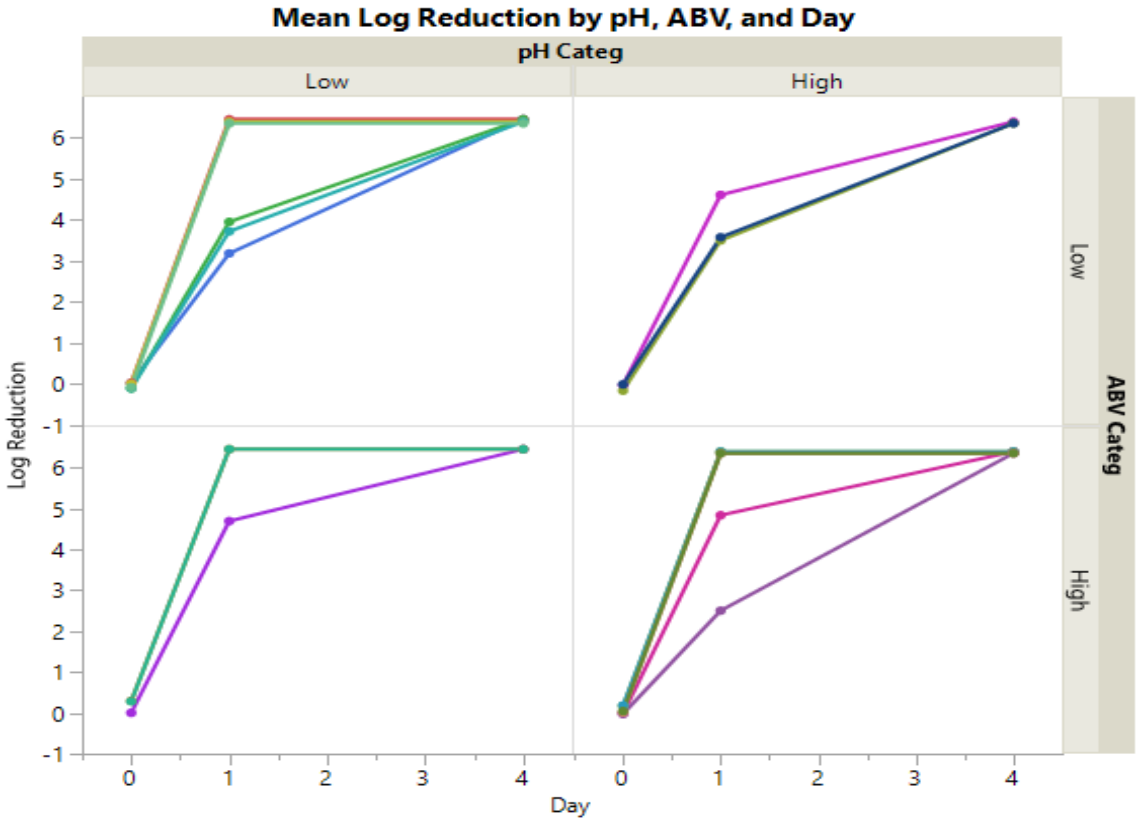

Figure G2. Extra visual image of data points graphed by pH, ABV, and Day for commercial cider data

Table G1. Fixed effects tests ran in JMP with repeated measures analysis of variance with commercial cider data

Fixed Effects Tests			
Source	Dfnum	F Ratio	Prob > F
Day	2	60859.7	<0.001
pHCat	1	16.9	0.0011
ABVCat	1	3.4	0.08
Day*ABVCat	2	5.7	0.01
MalicCat	1	1.1	0.32